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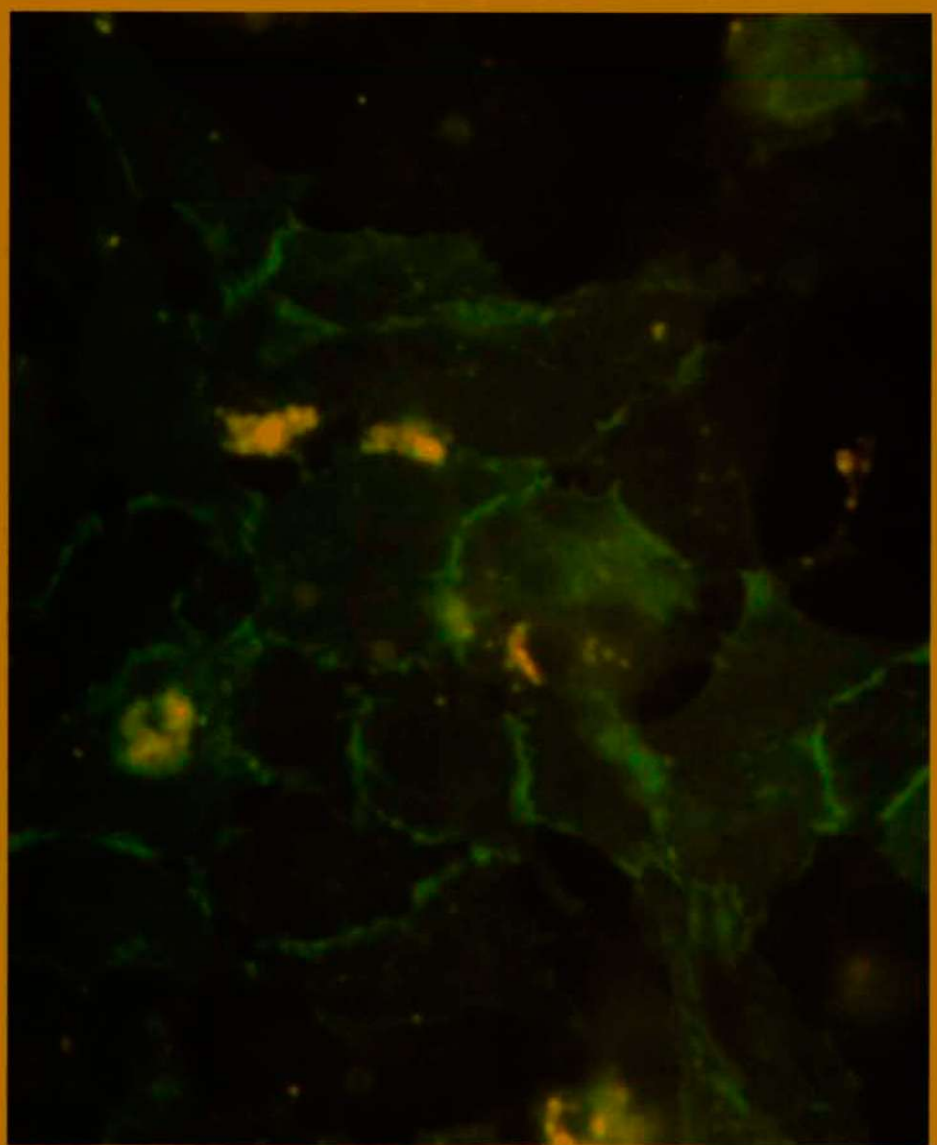

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ARTICLE

Comparison of treatment regimens to sensitize in situ hybridization for low-abundance calmodulin transcripts in the white matter of the rat spinal cord

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ABSTRACT We compared two in situ hybridization protocols for the detection of low-abundance calmodulin (CaM) I mRNA populations in the lipid-rich white matter of the rat lumbar spinal cord. Digoxigenin-labeled, CaM I gene-specific antisense and sense cRNA probes were used in these experiments. Although microwave heating followed by chloroform and 0.1% triton X-100 treatment resulted in the specific labeling of several cells in the gray matter of the spinal cord with a low nonspecific signal, it did not detect any CaM I expressing cells in the white matter. The protocol involving a hybridization solution adjusted to a slightly alkaline pH (pH 8.0), however, resulted in the detection of a large number of CaM-expressing cells not only in the gray matter, but also in the white matter of the spinal cord, with a nonspecific hybridization signal that was essentially identical to that of the background, and it also retained a much better overall tissue quality as compared with the protocol involving microwave heating and triton X-100 treatment. Numerous medium-sized astrocyte-like cells and smaller cells resembling oligodendrocytes were detected that expressed CaM I mRNAs throughout the white matter of the lumbar spinal cord. Thus, in situ hybridization carried out at a slightly alkaline pH is a far superior method for the detection of low-abundance mRNA populations in lipid-rich regions of the central nervous system, such as the white matter areas, as compared with microwave heating combined with triton X-100 treatment.
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KEY WORDS

alkaline pH
calmodulin
in situ hybridization
microwave
rat
spinal cord
triton X-100
white matter

In situ hybridization is widely used to study the spatiotemporal distribution of gene expression in nervous tissue. We are especially interested in the study of the distribution of calmodulin (CaM) mRNA populations in the rat brain under normal (Palfi et al. 1999) and experimental conditions (Palfi and Gulya 2001; Palfi et al. 2001; Vizi et al. 2000) by the use of in situ hybridization methods in which radioactive (Kortvely et al. 2002; Palfi et al. 1998, 1999) and digoxigenin (DIG)-labeled riboprobes (Kovacs and Gulya 2001, 2002) are used. The three CaM genes (CaM I, II and III) are widely expressed in the brain, where a widespread and differential area-specific distribution of the CaM mRNAs has been detected (Palfi et al. 1999). The expression patterns corresponding to the three CaM genes differed most considerably in the olfactory bulb, the cerebral and cerebellar cortices, the diagonal band, the suprachiasmatic and medial habenular nuclei, and the hippocampus. Moreover, the significantly higher CaM I and CaM III mRNA copy numbers than that of CaM II in the molecular layers of certain brain areas revealed a differential dendritic targeting of these mRNAs. CaM mRNA populations are abundant in the piriform cortex, the pyramidal and granular cell layers of the hippocampus, the amygdalohippocampal area, the nucleus of the lateral olfactory tract, the parafascicular thalamic nucleus, the superior colliculus, the pontine nuclei and the dorsal tegmental area. Low CaM mRNA populations are characteristic of the molecular (plexiform) layers of the olfactory bulb, the hippocampus and the cerebellum, and in general all white matter areas, including the anterior commissure, the cerebellar white matter, the corpus callosum, the internal capsule, the pyramidal tract and the ventral hippocampal commissure. Interestingly, there are no data on the CaM gene expression in the white matter of the spinal cord, possibly because of the very low amounts of CaM mRNAs in this area that are available for detection with conventional in situ hybridization methods, which possess only average sensitivity.

In our study, we explored the possibility that the lack of data regarding CaM gene expression in the white matter of the rat spinal cord is due to its generally very low expression level, and to the relative insensitivity of most current in situ hybridization protocols for low-abundance mRNA populations. Thus, the objective of the study was to localize CaM I mRNAs in the mature rat spinal cord white matter by using different in situ hybridization methods, involving either

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microwave heating combined with triton X-100 treatment or a slightly alkaline hybridization solution (pH 8.0) to increase the sensitivity, using CaM I gene-specific DIG-labeled antisense and sense cRNA probes in cryostat sections of the lumbar spinal cord of adult rats.

Materials and Methods

Experimental animals and tissue preparation

The experimental procedures were carried out in strict compliance with the European Communities Council Directive (86/609/EEC), and followed the Hungarian legislation requirements (XXVIII/1998 and 243/1998) regarding the care and use of laboratory animals. Adult (200-220 g) male Sprague-Dawley rats were maintained under standard housing conditions. The animals were killed by decapitation; their lumbar spinal cords were quickly removed, embedded in Cryomatrix embedding medium (Shandon Scientific Ltd., Pittsburgh, PA, USA) and frozen immediately at -70°C. Serial coronal cryostat sections (15 μm) were cut onto 3-aminopropyltriethoxysilane-coated glass slides, air-dried and stored at -70°C until further processing.

cRNA probes

For the preparation of DIG-labeled cRNA probes, in vitro RNA syntheses from the purified and linearized vectors were carried out to prepare antisense and sense CaM I cRNA probes, using a DIG RNA labeling kit (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturer's protocol, as described previously (Kovacs and Gulya 2001, 2002). Briefly, genomic sequences of the 3'-nonhomolog regions of CaM I, II and III mRNAs were amplified by polymerase chain reactions (PCRs). PCRs were performed by employing EcoR I and BamH I restriction enzyme cleavage site-extended primers. The primer sequences complementary to rat genomic DNA were as follows: for CaM I, 5'-AGACCTACTTTCAACTACT, corresponding to the 30-48-bp sequence, and 5'-TGTAAAACTCATGTAGGGG, corresponding to the 236-254-bp sequence of exon 6 (Nojima and Sokabe 1987); for CaM II, 5'-ATTAGGACTCCATTCCTCC, corresponding to the 144-162-bp sequence (numbered 1929-1947), and 5'-CACAACTCCACACTTCAACAGC, corresponding to the 353-374-bp sequence (numbered 2138-2159) of exon 5 (Nojima 1989); and for CaM III, 5'-ATGATGACTGCGAAGTGAAG, corresponding to the 12-31-bp sequence (numbered 7058-7077) of exon 6, and 5'-CAGGAGGAAGGAGAAAGAGC, corresponding to the sequence 153-172-bp downstream to the stop codon (numbered 7228-7247; Nojima 1989). Standard PCRs were run for 35 cycles (Palfi et al. 1998), and the resulting PCR products were cloned into a pcDNA3 vector (Invitrogen Corp., Carlsbad, CA, USA) and sequenced (AB 373 DNA Sequencer, PE Applied Biosystems, Foster City, CA, USA) to confirm their identity. In vitro RNA syntheses from the purified and linearized vectors were carried out to prepare antisense and sense cRNA probes. The complementary probe sequences were 225 bp (CaM I), 231 bp (CaM II) and 157 bp (CaM III) long. Labeled probes were purified by size exclusion chromatography on a ProbeQuant G-50 Sephadex micro column (Pharmacia Biotech, Uppsala, Sweden).

In situ hybridization

In situ hybridization of DIG-labeled cRNA probes was carried out in accordance with our previously described protocols (Kovacs and Gulya 2001, 2002). To check the effectiveness of microwave heating combined with triton X-100 treatment either with or without chloroform treatment, selected coronal cryostat lumbar spinal cord sections were heated by microwave irradiation (5 + 4 + 4 min, to prevent boiling) in 0.01 M Na-citrate solution (pH 6.0). The sections were either rinsed in chloroform for 5 min at room temperature (RT) and fixed for 5 min in 2x SSC containing 4% formaldehyde or were fixed immediately, then washed twice in 2x SSC for 1 min and rinsed in 0.1 M triethanolamine containing 0.25% acetic anhydride for 5 min at RT. At the end of the prehybridization procedure, the sections that underwent microwave heating were washed with either 0.1% or 1.0% triton X-100 in 2x SSC for 3 min. The sections were next washed in 2x SSC for 5 min, dehydrated, air-dried and hybridized in 50 μl hybridization solution (50% formamide, 4x SSPE, 1x Denhardt's reagent, 10% dextran sulfate, 100 mM DTT, 0.1% SDS, 100 μg/ml salmon sperm DNA and 100 μg/ml yeast tRNA) containing 200 ng/ml DIG-labeled probe. Hybridization was performed under parafilm coverslips in a humidified chamber at 55°C for 24 h. The pH of the hybridization solution was adjusted to either neutral (pH 7.4) or slightly alkaline (pH 8.0). The sections were rinsed in 2x SSC at RT and at 55°C for 5 and 10 min, respectively, and then treated with RNase A (16 μg/ml) at 37°C for 30 min. The sections were washed in 2x SSC/50% formamide at 55°C for 2 x 10 min, and in 2x SSC at 55°C and at RT for 10 min and 5 min, respectively. After posthybridization, the sections were washed in buffer B1 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 5 min, blocked in 5% heat-inactivated sheep serum in buffer B1 for 2 h and incubated in sheep anti-DIG-alkaline phosphatase conjugate (Boehringer Mannheim GmbH, Mannheim, Germany; 1:1000 dilution) in 5% sheep serum in buffer B1 at 4°C for 24 h. Sections were washed in buffer B1 for 3 x 5 min, and then in buffer B2 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 10 min, and were developed in buffer B2 containing 340 μg/ml nitro blue tetrazolium and 180 μg/ml 5-bromo-4-chloro-3-indolyl phosphate for 24 h under darkroom conditions. The color reaction was terminated by rinsing the sections in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA for 5 min at RT, and the

sections were then dehydrated and covered with glycerin/gelatin. Hybridized lumbar spinal cord sections were examined under a Leica DM LB microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany). Microscopic images (1600 x 1200 pixels, 8-bit gray scale) were captured with a Polaroid DMC 1 digital microscope camera (Polaroid, Cambridge, MA, USA) connected to a Power Macintosh computer (Apple Computer, Inc., Cupertino, CA, USA). Some of the sections were counterstained with toluidine blue or safranin.

Results

Microwave heating followed by triton X-100 treatment detected a number of CaM I mRNA-expressing cells in the gray matter of the lumbar spinal cord of the adult rat (Fig. 1A, C), irrespective of the chloroform treatment. Although most of the labeled cells were in the ventral horn, cells also expressed CaM I mRNA with lower frequency in the intermediate and dorsal horn. The concentration of triton X-100 used affected the number and intensity of the hybridization signal in the gray matter, as more intense labeling was apparent when the lower triton X-100 concentration was used (Fig. 1C). No specific hybridization signal was detected when the sense probe was applied at either triton X-100 concentration (Fig. 1B, D). However, no labeled cells were found in the white matter area of the spinal cord when the in situ hybridization protocols involving microwave heating and triton X-100 treatment were used to detect CaM I-expressing cells (Figs. 1A, C, and 2A, C).

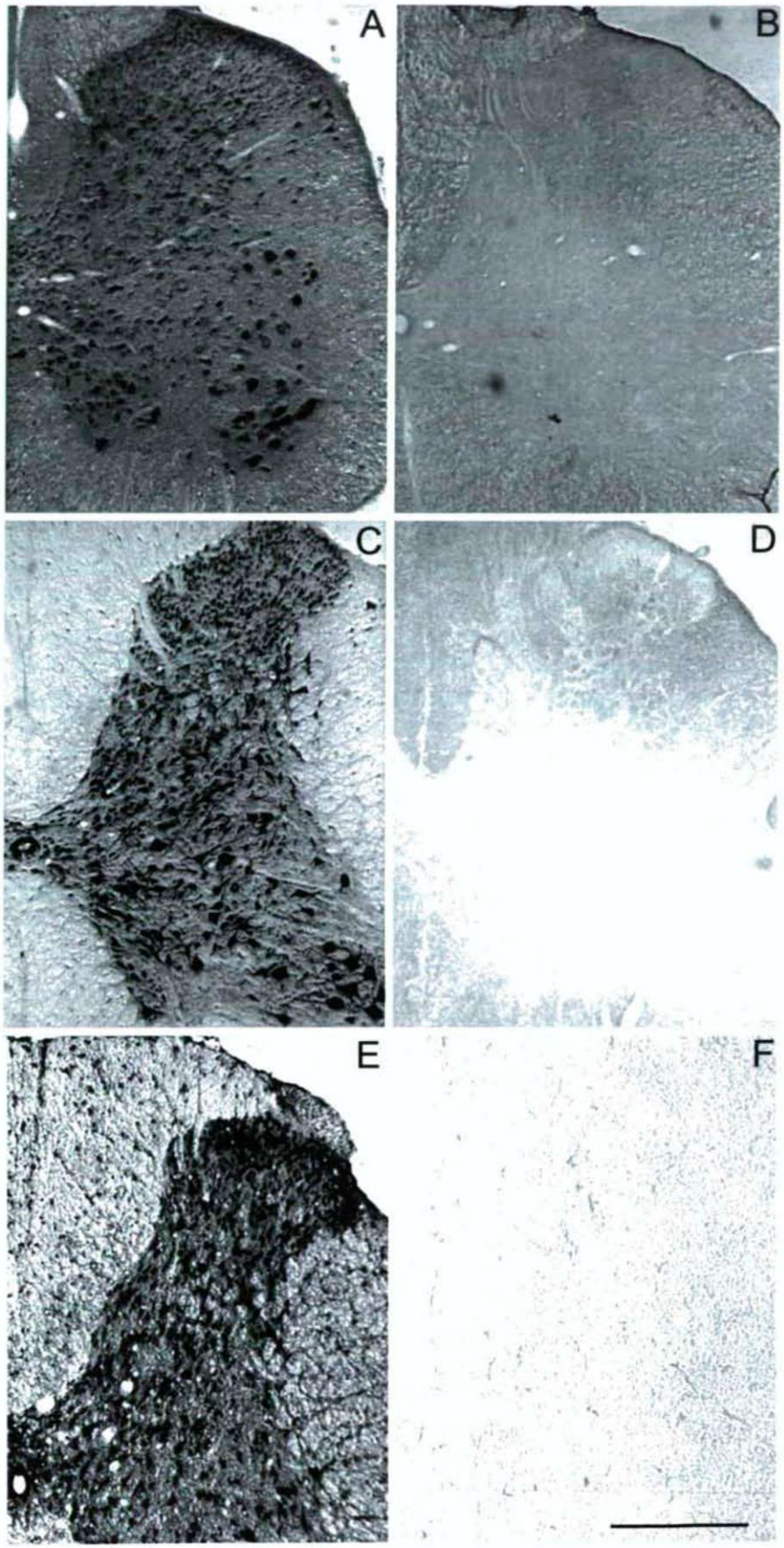
In a slightly alkaline hybridization solution, however, not only did the gray matter area of the spinal cord display numerous heavily labeled cells (Fig. 1E), but a large number of CaM I-expressing cells were also detected in the white matter area (Figs. 1E, and 2E). These cells were either medium-sized astrocyte-like cells, sometimes with short processes, or smaller cells resembling oligodendrocytes. The sense probe did not label any recognizable cellular elements, and resulted in a very faint staining (Figs. 1F, and 2F).

Discussion

White matter areas of the brain such as the anterior commissure, the corpus callosum, the internal capsule or the pyramidal tract contain very low amounts of CaM transcripts (Palfi et al. 1999), so the white matter of the spinal cord could likewise be very low in these mRNA populations. However, no data were available on the CaM-expressing cells in the white matter of the spinal cord by in situ hybridization, possibly because of its generally very low expression level and the relative insensitivity of most current in situ hybridization protocols for low-abundance mRNA populations. The high lipid content in myelin-rich regions of the nervous system could be a hindrance to the detection of mRNAs, and especially those populations that are in low abundance. Therefore, we speculated that the use of a more sensitive in situ hybridization method might successfully detect low-abundance CaM transcripts.

In recent years, several in situ hybridization techniques have been employed to increase the amount of detectable mRNAs or to sensitize the method for the detection of mRNA populations present in low abundance in the tissue. The amount of hybridizable mRNAs, for example, can be increased by either lipid removal or enzyme digestion with proteases, such as proteinase K (Relf et al. 2002), but enzymatic unmasking of the mRNA often occurs at the expense of tissue and cellular morphology (Lan et al. 1996). This has led to the extensive use of tissue heating methods such as microwave irradiation (Mitchell et al. 2001) or autoclaving (Eastwood and Harrison 1999; Oliver et al. 1997). Autoclaving proved to be superior to microwave heating because the latter method often results in local superheating of the tissue or evaporation of the buffer solution during irradiation in both immunocytochemical and in situ hybridization protocols (Norton et al. 1994; Oliver et al. 1999). While autoclaving can indeed increase the amount of hybridizable mRNA, especially in paraffin-embedded sections, other means of tissue preparation, such as the use of frozen sections, did not benefit from this procedure. A quantitative study confirmed that autoclaving is a suitable procedure that allows formalin-fixed, paraffin-embedded nervous tissue to be used for in situ hybridization, as the signal intensity was stronger (by 35–50%, in terms of nCi/g tissue equivalents) in autoclaved sections than in frozen sections (Eastwood and Harrison 1999).

Our in situ hybridization protocol at a slightly alkaline pH overcomes this problem and it is superior to other techniques in which tissue heating is combined with triton X-100 treatment. Basyuk et al. (2000) introduced alkaline fixation to in situ hybridization techniques in order to improve the sensitivity. They reported that alkaline formaldehyde (pH 9.5) dramatically increased (5-6-fold) the in situ hybridization signal with riboprobes for the detection of both low- and high-abundance mRNAs. Since alkaline fixation does not improve the retention of mRNA during in situ hybridization (Basyuk et al. 2000), but rather increases the accessibility of the target for the riboprobe, hybridization at alkaline pH is crucial for the increased sensitivity. Our protocol was successfully used for DIG-labeled CaM gene-specific riboprobes on both cryostat sections of the spinal cord (Kovacs and Gulya 2001, 2002) and paraffin sections of the retina (Kovacs and Gulya 2003), i.e. tissues with either very high or very low lipid content, but both with very low CaM mRNA abundance.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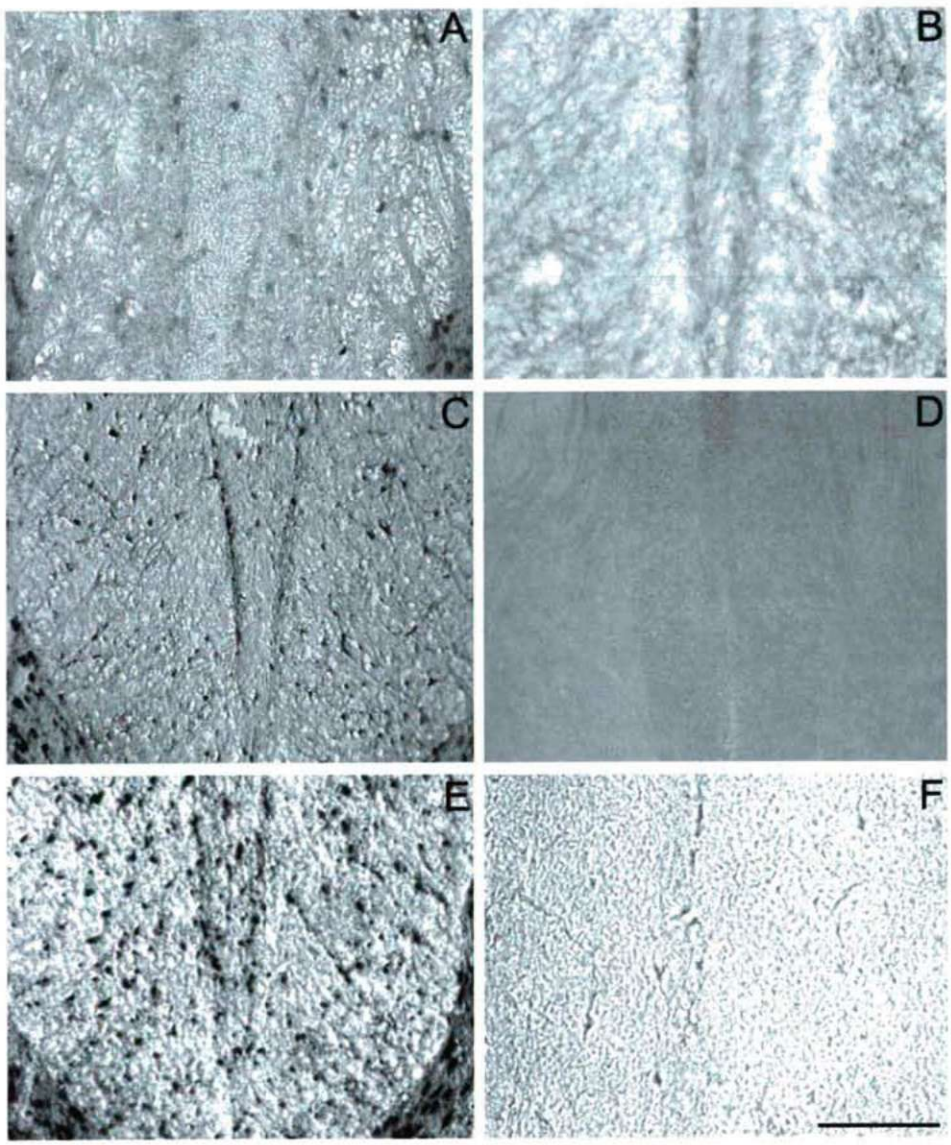

Figure 2. Hybridization of DIG-labeled antisense (A, C, E) and sense (B, D, F) cRNA probes specific for CaM I mRNAs in cryostat sections of the dorsal white matter of the adult rat lumbar spinal cord. The sections underwent the following treatments: A, B: microwave heating (5 + 4 + 4 min) before the immersion fixation, and 1.0% triton X-100/2x SSC treatment for 3 min at the end of prehybridization; C, D: microwave heating (5 + 4 + 4 min) before chloroform treatment and immersion fixation, and 0.1% triton X-100/2x SSC treatment for 3 min at the end of prehybridization; E, F: hybridization in a slightly alkaline solution (pH 8.0). Hybridization with the sense probes resulted in a very low signal in all cases (B, D, F). Microwave heating and triton X-100 treatment resulted in no labeled cells in the dorsal column (A). The highest number of labeled cells and the strongest signal in the dorsal column of the white matter could be detected after hybridization in a slightly alkaline solution (E). The CaM I-expressing cells, sometimes with their processes, could be easily visualized. Portions of the gray matter can be seen in the lower corners of the pictures in A, C, E. Scale: 200 μm.

Figure 1. Hybridization of DIG-labeled antisense (A, C, E) and sense (B, D, F) cRNA probes specific for CaM I mRNAs in cryostat sections of the adult rat lumbar spinal cord. The sections underwent the following treatments: A, B: microwave heating (5 + 4 + 4 min) before immersion fixation, and 1.0% triton X-100/2x SSC treatment for 3 min at the end of prehybridization; C, D: microwave heating (5 + 4 + 4 min) before chloroform treatment and immersion fixation, and 0.1% triton X-100/2x SSC treatment for 3 min at the end of prehybridization; E, F: hybridization in a slightly alkaline solution (pH 8.0). Microwave heating and treatment with 1.0% triton X-100 resulted in relatively high nonspecific labeling in both the gray and the white matter areas. CaM-expressing cells could be detected only in the gray matter. The neuropil displayed a relatively strong, diffuse signal. Microwave heating and treatment with 0.1% triton X-100 resulted in a better tissue quality with a lower nonspecific background. Occasionally, the cells in the gray matter exhibited their processes (C). No labeled cells could be seen in the white matter of the spinal cord (D). When the hybridization took place in a solution adjusted to a slightly alkaline pH, a large number of cells demonstrated CaM I expression not only in the gray matter, but also in the white matter of the spinal cord (E). The cells in the ventral horn were heavily labeled, while the cells in the intermediate and dorsal horn were less intensely labeled. Hybridization with the sense probe resulted in a very low signal (F). Scale: 500 μm.

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ARTICLE

Lipid rafts in the plant plasma membrane?

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ABSTRACT In order to study the molecular characteristics of an integral membrane protein, first the protein should be solubilized. Ascorbate-reducible *b*-type cytochromes are highly hydrophobic integral membrane proteins with six trans-membrane α-helices. A fully ascorbate-reducible *b*-type cytochrome was easily solubilized by Triton X-100 from phase partition-purified plasma membranes of 5-day-old etiolated bean (*Phaseolus vulgaris* L.) hooks (Trost et al. 2000). However, under the very same conditions, a similar protein in the phase partition-purified plasma membrane of 9-week-old green *Arabidopsis thaliana* leaves seemed to be very resistant to solubilization with Triton X-100 (Bérczi et al. 2001). It was assumed that the composition of lipids in the plasma membrane of the two different tissues might influence the solubilization. Results obtained with thin layer chromatography revealed that both quantitative and qualitative differences exist between the lipid composition of the two plant plasma membranes. The observations are discussed in the light of possible existence of "lipid rafts" in the plant plasma membranes.
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KEY WORDS

lipids
plant plasma membrane
rafts
solubilization
Triton X-100

Integral membrane proteins with trans-membrane localization play crucial roles in biological transport processes. Numerous members of a newly-recognized protein family, the cytochromes *b*-561 (cyts *b*-561), function as electron transporters both in animal and plant membranes (Asard et al. 2000, 2001). These proteins are highly hydrophobic, fully ascorbate-reducible, have 6 trans-membrane α-helices, and transport electrons through different biological membranes. Presence of *b*-type cytochromes in plant plasma membranes (PM) were first demonstrated in the microsomal fraction from etiolated corn coleoptiles (Jasaitis et al. 1977) then, together with other *b*-type cytochromes, in the highly-purified PM vesicles from different plant tissues (Asard et al. 1989; Askerlund et al. 1989). Purification and partial characterization of an ascorbate-reducible *b*-type cytochrome (asc-red. cyt *b*) from the phase partition-purified PM was first successful from etiolated bean hypocotyl hooks by solubilization with Triton X-100 as detergent (Trost et al. 2000). When a similar protein was tried to be solubilized, under identical conditions, from the phase partition-purified PM of 9-week-old green *Arabidopsis thaliana* leaves as well as from that of 5-day-old corn (*Zea mays* L.) roots, the PM proteins seemed to be very resistant to Triton X-100 (Bérczi et al. 2001).

It has long been known that lipids occur in several states in lipid bilayers as well as in biological membranes. The two most well-known states (or phases) are the "lamellar liquid crystalline" (L_α) and the "lamellar gel" (L_β) phases (Caffrey and Cheng 1995; Nagle and Tristram-Nagle 2000; Simons and Toomre 2000). Recent work suggests that lipids in biological membranes may also exist in a third phase (or physico-chemical state) that may be of biological significance; it is the "liquid ordered" phase (L_o) with properties intermediate between L_α and L_β (Brown and London 1998). It has also been shown that membrane fractions in L_o phase, which are enriched in sphingolipids and sterols (ST), are insoluble with non-ionic detergents (*i.e.* Triton X-100); these microdomains are called "lipid rafts" (Simons and Ikonen 1997; Rietveld and Simons 1998; London and Brown 2000). Lipid rafts containing a given set of proteins (*i.e.* heterotrimeric G-proteins and their receptors, GPI-anchored proteins, etc.) can change their size and composition in response to different stimuli, among them to solubilization too. Although there is a remarkably large proportion of lipids, with potential to form rafts, present in both the animal cell membrane (Mayor and Maxfield 1995) and the plant plasma membrane (Rochester et al. 1987; Uemura and Steponkus 1994), occurrence and analysis of lipid rafts in plant plasma membranes has rarely been reported (Peskan et al. 2000). It is possible that the different capability of Triton X-100 in solubilizing the asc-red. cyts *b* from different tissues refers to the presence of different amount of special lipids and sterols participating in formation of liquid-ordered and liquid-disordered phase domains (lipid rafts) in the plant PM too. This hypothesis is strongly supported by the results of lipid analysis of corn (*Zea mays* L.) root PM vesicles (Bohn et al. 2001).

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In the present short paper, experimental evidence is presented for the different composition of PM vesicles purified from etiolated *Phaseolus* hypocotyl hooks and green *Arabidopsis* leaves. Thin layer chromatography (TLC) analysis of total lipids extracted from the tissues revealed that sterols and cerebrosides (CER), the two major and potent compounds of lipid rafts, are present in much higher amount in the PM from green *Arabidopsis* leaves than in that from etiolated *Phaseolus* hooks.

Materials and Methods

Chemicals

Standards for phospholipids [PC (1,2 diacyl-sn-glycero-3-phosphocholine); PE (1,2-dicayl-sn-glycero-3-phosphoethanolamine); PG (1,2-diacyl-sn-glycero-3-phospho-(1-rac-glycerol)); PI (phosphatidylinositol 1,2-diacyl-sn-glycero-3-phospho-(1-D-myo-inositol)); PS (1,2-diacyl-sn-glycero-3-phospho-L-serine)] as well as standards for sterols (Stigmasterol, Campesterol, β-sitosterol) were purchased from Sigma-Aldrich. Standards for sphingolipids (Glucoserebrosides, Ceramide, Phytosphinanine, D-erythro-sphinganine) were ordered at Avanti Polar Lipids (USA). Solvents were of HPLC-grade and purchased from Lab-Scan analytical sciences (Belgium).

Plant material and PM preparation

Etiolated seedlings of bean (*Phaseolus vulgaris* L. cv. Limburgse Vroege) were grown for 5 days in the dark on moist vermiculite at 25°C. Hypocotyl hooks (150 g fresh weight) were harvested on ice and used for PM preparations (Asard and Bérczi 1998).

Arabidopsis thaliana (L.) Heynh (ecotype Columbia) was grown as described earlier (Bérczi et al. 2001). Leaves of 9-week-old seedlings (seedlings just before flowering) were harvested and immediately used for PM preparation (Bérczi et al. 2003).

PM vesicles were prepared by aqueous polymer two-phase partitioning (Larsson et al. 1994); the final PM-rich upper phases (U_3+U_3') were diluted 6-fold by 10 mM Tris-KOH buffer, pH 8 and pelleted by centrifugation at 50,000 g_{max} for 90 min. Pelleted PM vesicles were re-suspended in 10 mM Tris-HCl, pH 8, containing 1% (w/v) glycerol and stored at -75°C until use.

Stripping and solubilization

PM vesicles from two-three preparations (2-3 times 4 ml) were taken from the deep-freezer, them thaw up at room temperature and combined to obtain 12-15 mg PM protein. Loosely-bound proteins and proteins trapped in the lumen of sealed vesicles were removed by a one-step stripping protocol as follows. The 12 ml solution of PM vesicles was first diluted to 50 ml by addition of 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, then 2 µl of nonaethylene glycol monododecyl ether ($C_{12}E_9$, a non-ionic detergent) were added (final detergent concentration was below the critical micellar concentration of $C_{12}E_9$) and the PM vesicles were incubated with gently but continuous stirring at room temperature for 20 min. After 20 min 5.5 ml of 5 M KCl was added (final KCl concentration of 0.5 M) and the PM vesicles were further incubated for 40 min. The so called "stripped" PM vesicles were pelleted at 100,000 g_{max} and 4°C for 45 min. The pellet was re-suspended in 10 mM Tris-HCl, pH 8.0, 1% (w/v) glycerol and stored at –75°C until use.

Lipid extraction and thin layer chromatography

Total lipids were extracted from stripped PM vesicles as described by Bligh and Dyer (1959) with minor modifications (Bohn et al. 2001). Briefly, vesicles with 1 mg of protein from both PM preparations were washed in 0.5 M KCl, 1 mM Na_2-EDTA and pelleted at 100,000 g_{max} and 4°C for 60 min. Each pellet was resuspended in 1 ml ion-exchanged (Milli-Q) water. To the 1 ml suspension 3.75 ml of chloroform:methanol (2:1, v/v) were added and rigorously vortexed for 10 min. Then 1.25 ml chloroform was added and vortexed for 1 min and finally 1.25 ml of 1 M NaCl was added and again vortexed for 1 min. Phase separation was accomplished by centrifugation at 5,000 g_{max} and 4°C for 10 min. The lower phase was collected with a Pasteur pipette. After evaporation of the organic solvents, the lipid extract was dissolved in 100 µl of chloroform and used immediately for TLC.

Major lipid classes were separated by two dimensional TLC on silica gel (DC-Platten 20x20 cm Kieselgel 60, Merck KGaA, Darmstadt, Germany) as described by Kates (1972) with minor modifications. Briefly, plates had been heated at 100°C for 12 h before use. For spotting, 25 µl of lipid extract was used. Separation was by chloroform-methanol-18% ammonia (65:35:5, v/v; first direction) and then by chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1, v/v; second direction). Phosphatidic acid (PA), phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG), phosphatidyl inositol (PI), phosphatidyl serine (PS), cerebrosides and sterols were run one-by-one under identical conditions on separate plates as standards. Lipids were detected in a chromatographic tank saturated with iodine vapor for 10 min. Developed plates were immediately scanned by a Umax Powerlook 2000 scanner at 400 dpi. Images were processed with Adobe Photoshop. Spots were analyzed with OptiQuant version 02.50 software (Packard Instrument Co., Meriden, CT, USA).

Results presented are averages from four independent experiments and eight 2D-TLC separations. T-test (P=0.01) was used to confirm significance.

Results and Discussion

It has recently been shown that the solubilization yield of proteins from PM vesicles purified from *Arabidopsis* leaves and corn roots is much lower (about 35%) than that from bean hypocotyl hooks (about 95%; Bérczi et al. 2001). It has also recently been shown that corn root PM contains about as much free sterols (40.8 mol%) as phospholipids (43.9 mol%) in the PM. These results are in agreement with earlier lipid analysis results for the corn root PM (Grandmougin et al. 1989; Cowan et al. 1993). Also, lipid analysis results for leaf PM from different species revealed the presence in high molar percentage of free sterols and cerebrosides (Rochester et al. 1987; Uemura and Steponkus 1994). Literature data thus support the hypothesis that the low solubility of integral PM proteins with Triton X-100 from corn roots and *Arabidopsis* leaves might be due to the presence of lipid compounds capable of formation of insoluble lipid microdomains.

In the light of the data in literature it seems that it is not the low solubility of PM proteins from *Arabidopsis* leaves and corn roots but rather the high solubility of PM proteins from bean hooks is exceptional. Would it be supported by the lipid composition of bean hook PM? In order to answer the question total lipids from both bean hook and *Arabidopsis* leaf PM were extracted and analyzed for the major phospholipid compounds, cerebrosides and for sterols by 2D-TLC (Fig. 1) and densitometry (Fig. 2).

According to Fig. 2, no significant changes can be found in the phospholipid composition of PM vesicles isolated from etiolated bean hypocotyl hooks and Arabidopsis leaves. Practically, both PM is composed of 34% PA, 23%PC, 23% PE, 11% PS, 6% PI and traces of PG. When specific staining for phospholipids was performed (phosphate staining), results were similar. However, significant differences were found for the spots representing FFA, CER and ST. FFA were present in slightly higher amounts in the PM of bean hooks. CER are present in higher concentrations in the PM of Arabidopsis leaves, roughly twice the amount detected in the PM of etiolated bean hypocotyl hooks. Care must be taken interpreting the result as CER was measured as an area where cerebrosides were found under the 2D-TLC conditions applied. It means that co-localization of other unknown

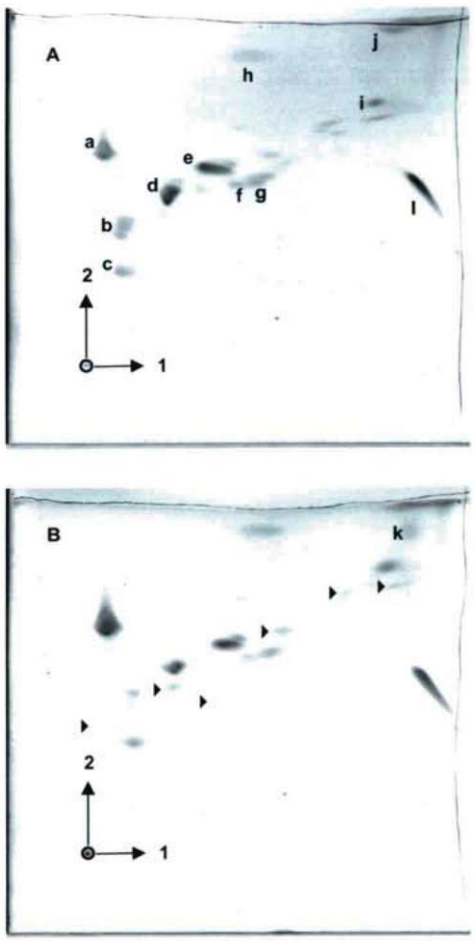

Figure 1. Two dimensional TLC of total lipid extracts from PM purified from etiolated bean hypocotyl hooks (A) and from green *Arabidopsis* leaves (B). Numbers 1 and 2 refer to the first and second directions in separation. Separation was by chloroform-methanol-18% ammonia (65:35:5, v/v; direction 1) and then by chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1, v/v; direction 2). Lipids were detected with iodine vapor for 10 min. Identification of spots were by running and developing standards under identical conditions. Standards were phospatidic acid (a), phosphatidyl serine (b), phosphatidyl inositol (c), phosphatidyl coline (d), phosphatidyl ethanolamine (e), phosphatidyl glycerol (f), cerebrosides (g), and sterols (i+ k). Free fatty acids (FFA; h) and neutral lipids (j) were identified on the basis of literature (Kates 1972). Identity of a major compound (l) could not be determined with this technique. Minor spots with significantly different intensity on the two plates are labeled with arrow-heads; their identification awaits for further studies.

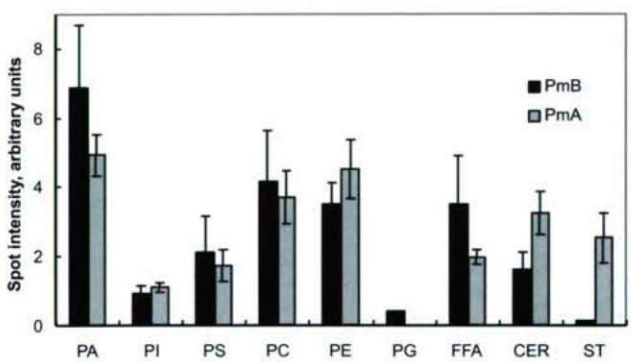

Figure 2. Distribution of lipids in the PM from etiolated bean hypocotyl hooks (black columns, PmB) and green *Arabidopsis* leaves (gray columns, PmA). PA, phospatidic acid; PI, phosphatidyl inositol; PS, phosphatidyl serine; PC, phosphatidyl coline; PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol; FFA, free fatty acids; CER, cerebrosides; ST, sterols. Columns marked with an asterix represent significant differences (P = 0.01).

compounds cannot be excluded. Amount of STs were about tenfold higher in the PM from *Arabidopsis* leaves as compared to that from etiolated bean hypocotyl hooks. However, the overall sterol concentrations appear to be rather low as compared to literature data. This might be due to the lower response of sterols to iodine staining. A specific sterol staining however proved that the amount of sterols in PM from *Arabidopsis* leaves was in agreement with literature data but that from etiolated bean hypocotyl hooks was significantly less. Sterol results are further clouded by the problem of separating them from NL in some cases.

Since the amount of CER and ST (1) appears to give the most significant differences in the composition of PMs from the two sources and (2) CER and ST are the most important lipid components for raft formation, future experiments will focus on more accurate determination of these compounds (by HPLC with ELSD detection).

In summary, it can be concluded that (1) the relative low abundance of sterols and some lipid compounds in the bean hook PM, as compared to the *Arabidopsis* leaf PM as well as to other leaf and root PM published in the literature, might provide proper condition for the high solubility of this PM by Triton X-100, and (2) low solubility of plant PM with Triton X-100 can be the consequence of formation of lipid rafts insoluble by nonionic detergents, like it is so in the case of animal cell membranes.

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ARTICLE

Ocadaic acid treatment alters the intracellular localization of caveolin-1 and caveolin-2 in HepG2 cells

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ABSTRACT In this paper we provide evidences that protein phosphatases could regulate the intracellular localization of caveolin isoforms in a hepatoma cell line (HepG2). Ocadaic acid (OA) - a serine/threonine phosphatase inhibitor – was used in various concentrations (4nM and 100nM) to study the localization of caveolin-1 and caveolin-2 in HepG2 cells. Using fluorescent and confocal immunocytochemistry we have found that OA in both concentrations has significantly altered the intracellular localization and distribution of the caveolin-1 and caveolin-2 as well. In control (-OA treatment) the caveolin-1 was present in discrete punctate structures in the cytoplasm and also on the cell membrane. Caveolin-2 has partly overlapped with caveolin-1, but a significant amount caveolin-2 was detected around the nucleus. After OA (4 and 100 nM) treatment caveolin-1 has disappeared from the cell membrane, it was present mainly in the cytoplasm in larger vesicle or vacuole-like structures that were arranged along the cables of the cytoskeleton. In many cases caveolin-2 was found to colocalize with caveolin-1, but there was always a significant amount of caveolin-2 present around the nucleus. Immunoprecipitation and Western blot analysis revealed that in OA-treated cells a ~24 kDa protein identified as caveolin-2 was strongly phosphorylated on tyrosine residues. The effect of OA was not reversible, since the removal of OA has not resulted in the dephosphorylation of caveolin-2 and the perinuclear localization of caveolin-2 remained. Our data indicate that phophorylation of caveolin-2 can alter not only the intracellular localization of caveolin isoforms but also the distribution of caveolae. The cytoskeleton seems to play an important role in the normal and altered distribution of caveolae, and the tyrosine phosphorylation or the absence of dephosphorylation of caveolin-2 isoform can inhibit the recycling of caveolae.
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KEY WORDS

caveolae-cycle
tyrosine phosphorylation
caveolin-2
resident and elicited macrophages
phosphatase inhibitors

Caveolae have been morphologically characterized as omega- or flask-shaped plasma membrane invaginations and biochemically as caveolin- and cholesterol-rich membrane domains (Kurchalia and Parton 1999). Caveolins are essential for caveolae formation, they constitute the structural framework of caveolae (Parton 1996). Three members of the caveolin gene family have been identified so far: caveolin-1, caveolin-2 and caveolin-3. Caveolin-1 induces caveolae formation, binds cholesterol, and interacts with signaling molecules. Caveolin-3, expressed in limited kinds of cells (muscle cells, Tang et al. 1994; Song et al. 1996; Way and Parton 1999; glials cells, Nishiyama et al. 1999; neurons, unpublished data, etc.), is thought to play similar role to that of caveolin-1. The function of caveolin-2 has not yet been defined in details. Data indicate its accessory role in caveolae formation, as well as its involvement in forming deep caveolae invaginations (Scheiffele et al. 1998; Fujimoto et al. 2000; Kiss et al. 2000).

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Caveolae seem to have multiple functions. Wide variety of signaling molecules (GPI-anchored proteins, intermediates of the MAP kinase phosphorylation cascade including h-Ras, Fyn, Src family tyrosine kinases, eNOS, heterotrimeric G proteins, G-protein-coupled receptors) have been found to be accumulated in caveolae (Anderson, 1998; Smart et al. 1999). These signaling molecules bind to caveolin itself (Couet et al. 1997). As a result of this binding the signaling molecules become inactive (Li et al. 1995), thus caveolae assumed to function as preassembled signaling complexes, message centers, signaling organelles (Smart et al. 1999). Caveolae also play an important role in the regulation of cellular cholesterol homeostasis (Fielding and Fielding 2000).

Caveolae take part in cellular transport as well. Increasing number of evidences confirm that caveolae are directly involved in the internalization of membrane components, extracellular ligands such as cholera toxin (Montesano et al. 1982; Parton et al. 1994), folic acid (Rothberg et al. 1990; Anderson et al. 1992), serum albumin (Scnitzer et al. 1994),

autocrine motility factor-AMF (Berliname et al. 1998), GPI-anchored proteins (Anderson 1992) green fluorescent protein (Nichols et al. 2001), urokinase receptors (Stahl amd Mueller 1995). Certain filamentous adhesin (FimH)-expressing bacteria are also internalized in caveolae-dependent pathway in immune cells (reviewed by Harris et al. 2002). Several nonenveloped viruses (Simian virus 40, Polyoma virus) enter cells through caveolae (Pelkmans et al. 2001; Pelkmans and Helenius 2002).

It has been postulated that phosphatases and kinases can regulate the internalization, and probably, the recycling of caveolae. In our experiments ocadaic acid (OA) was used to study the internalization and possible recycling of caveolae in HepG2 cell line. When hepatoma cells were treated with OA (4 and 100 nM), the cytoplasmic and plasma membrane distributions of caveolin-1 and caveolin-2 have changed. Caveolin-1 has disappeared from the cell surface and it was detected in vesicles arranged in rows in the cytoplasm. Since caveolae can be characterized by the presence of caveolin-1 and caveolin-2 (caveolin-2 seems to play an accessory role in caveolae-formation), the cytoplasmic route of caveolae can be followed by detection (localization) of these isoforms, so we think that caveolae have also disappeared from the cell surface. Although caveolin-2 was partly colocalized with caveolin-1, there was a significant amount of this isoform present in the perinuclear region. When OA was removed and the cells were incubated with an OA-free medium for 3 hours caveolin-1 was still arranged along cables, while caveolin-2 was detected around the nucleus. This perinuclear caveolin-2-containing ring was more prominent then in control cells. We were not able to detect caveolin-1 and –2 in punctate structures on the cell membrane. Our immunoprecipitation and Western blot analyses showed that OA treatment has resulted in tyrosine phosphorylation of a ~24 kDa caveolin-2. When the cells were incubated with OA-free medium for 3 hours, the tyrosine phosphorylation of caveolin-2 became stronger indicating that the effect of OA was not reversible. From these data we conclude that tyrosine phosphorylation of caveolin-2 plays an important role in the regulation of caveolin and caveolae distribution/localization in the cytoplasm. Cytoskeleton seems to be involved in this procedure.

Materials and Methods

Materials

HepG2 hepatoma cell line was used for all experiments. The monoclonal antibodies (anti-caveolin-1 and anti-caveolin-2) were purchased from Transduction Laboratories, (Lexington, KI, USA). Anti-caveolin-1N, anti-caveolin-2 antibodies as well as anti-phosphotyrosine caveolin-1 have been purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibody against phosphotyrosine (4G40) was obtained from Upstate Biotechnology. The horseradish peroxidase-conjugated (HRP) anti-mouse and anti-rabbit IgG and the prestained standard protein markers were Bio-Rad (Hercules, CA, USA) products. The ECL nitrocellulose filter and the ECL reagent were manufactured by Amersham Bioscience Trading GmbH (Vienna, Austria). Protein phos-phatase 2A1 and protein tyrosine phosphatase (LAR) were purchased from Calbiochem (Lucerne, Switzerland).

Biotinylated anti-mouse IgG was obtained from Vector Laboratories (Burlingame, CA, USA), Alexa-conjugated anti-rabbit antibody (488 nm) and Alexa-conjugated avidin (594 nm) were purchased from Molecular Probes (Eugene, OR, USA).

Western blot analysis

HepG2 cells were solubilized with 1% SDS in 20 mM TRIS-HCL buffer, (pH 7.4) and boiled for 4-5 min. The protein contents of the lysates were measured with Lowry's method. 10-30 mg protein was separated on gradient (8-12% acrylamide) SDS-PAGE and transferred to nitrocellulose filters. The filters were blocked with 3% BSA (dissolved in 0.1% Tween-PBS). Incubation was carried out with anti-caveolin-1 (VIP21) IgG (1:250), or anti-caveolin-2 (1:250) antibody. The second antibody (anti-rabbit IgG-HRP) was diluted to 1:10,000 (for anti-caveolin-1) and 1:3,000 (for anti-caveolin-2). The conditions of immunoblotting (incubation time, washing, ECL detection) were chosen as suggested by the manufacturer (Amersham Bioscience). After ECL detection the results were evaluated with an LKB Laser Densitometer using the GelScan program.

Immunoprecipitation

Cells (10^7) were lysed in 200 ml solubilization buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 10% glycerol, 0.5% Nonidet P40, 0.1 mM PMSF, 10 mg/ml aprotinin). The lysates were incubated with specific antibodies, anti-caveolin-1 and anti-caveolin-2, for 5 h at 4°C. Immune complexes were formed by addition of protein-A-Sepharose 4B and incubated for 1 h at 4°C. The immune complexes were then sedimented by centrifugation at 12,000 g, followed by 4-5 washes in lysis buffer. Bound proteins were solubilized and analyzed on SDS-PAGE, follwed by immunoblotting. In some experimental groups the immunoprecipitates were treated with tyrosine phophatase (15 min at 25°C) dissolved in a buffer containing 20 mM Tris-HCL and 1 mM EDTA (pH 7.4).

Immunocytochemistry

HepG2 cells were fixed in methanol-aceton mixture at -20°C. After fixation endogenous biotin was blocked, then the cells were washed. To prevent aspecific binding the cells were treated with 1% BSA/PBS and incubated with anti-caveolin-1 (1:200) and anti-caveolin-2 (1:100) antibodies for over-

night. After washig (3 times in 0.1% Triton-X 100 containing PBS) anti-caveolin-1 was detected with Alexa-conjugated anti-rabbit IgG (488 nm, 1:100) To detect caveolin-2 a second antibody (biotinylated anti-mouse IgG in 1:100 dilution) was used and it was visualized with Alexa-conjugated avidin (594 nm, 1:100). Fluoromont was used to cover the cells. The samples were studied with Zeiss Axiophot microscope and with MRC 1024 Bio-Rad confocal scanning microscope.

Results

The effect of ocadaic acid on the intracellular distribution of caveolin-1 and caveolin-2 isoforms

When we used fluorescent and confocal immunocytochemistry we have found that OA in both 4 and 100nM concentrations has significant effect on the intracellular distribution of the caveolin-1 and caveolin-2 as well. In control (-OA treatment) the caveolin-1 was found in small punctate structures on the plasma membrane, but it was also detectable all over the cytoplasm. (Fig.1b) There was no preferential localization or cluster of these isoform. Caveolin-2 showed some colocalization with caveolin-1, but a significant amount caveolin-2 was detected around the nucleus (Fig 1c). After OA (4 and 100 nM) treatment caveolin-1 has disappeared from the cell membrane, it was present mainly in the cytoplasm in larger vesicles or vacuole-like structures (Fig. 2b and Fig. 3b). Most of these vesicles were arranged in rows, along cable-like structures suggesting that the cytoskeleton must be involved in remodelling of the caveolin distribution pattern. Although in many cases caveolin-2 was found to colocalize with caveolin-1, there was always a significant amount of caveolin-2 present around the nucleus (Fig. 2c and Fig 3c). When OA was removed and the cells were incubated for 3 hours in an OA-free culture medium the perinuclear localization of caveolin-2 remained (Fig 4c and Fig 5c) while caveolin-1 was found to follow the arrangement of the cytoskeleton. (Fig 4b and Fig 5b).

The effect of ocadaic acid on the phosphorylation of caveolin isoforms

When we immunoprecipitate proteins from the lysate of HepG2 cells with anti-caveolin-2 antibody a 24 kDa protein was the only one immunoprecipited with this antibody. When we used anti-caveolin-2 antibody this 24 kDa band showed a strong labelling indicating that this protein is caveolin-2. Our Western blot analysis revealed that in OA-treated cells this 24 kDa protein (caveolin-2) was strongly phosphorylated on tyrosine residues (Fig 6 H/4 and H/100), giving evidence that OA treatment induces a strong tyrosine phosphorylation of caveolin-2 in HepG2 cells. The effect of OA was not reversible, since the removal of OA has not resulted in the dephosphorylation of caveolin-2 (Fog 6 RH/4 and RH/100).

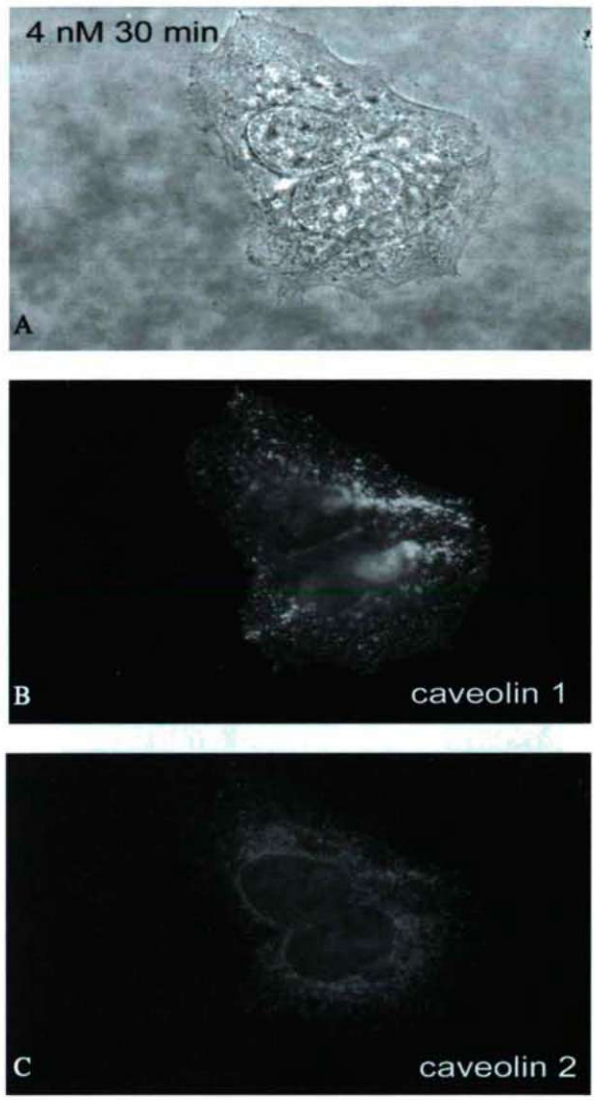

Figure1. The intracellular localization of caveolin-1 (**B**) and caveolin-2 (**C**) in control (no OA treatment cells. (**A**) phase contrast micrograph of a HepG2 cell. Caveolin-1 was found on discrete punctate structures on the cell surface and also in the cytoplasm (**B**). Caveolin-2 has partly overlapped with caveolin-1, but there is a preferential localization around the nucleus (**B**). Magnification: 1000x

Discussion

It has been generally accepted that besides clathrin-coated uptake mechanism alternative endocytotic pathways also exist. There are several candidates by which this alternative uptake can occur, one of them is the endocytosis via caveolae. Although it has been debated for a long time that caveolae can pinch off from the plasma membrane to form primary endocytotic vesicles (Anderson 1993; Van Deurs et al. 1993), there is an increasing body of evidence confirming that caveolae do pinch off from the plasma membrane and are

directly involved in the internalization of membrane component, extracellular ligands (Parton et al. 1994; Schnitzer et al. 1994; Berliname et al. 1998; Monteasno et al. 1998; Nichols et al. 2001; Stahl and Mueller 1995).

The caveolae-mediated endocytosis differs from clathrin-mediated pathway in many respects. In contrast to clathrin-coated endocytosis, the internalization of caveolae seems to be regulated by kinases and phosphatases. The increased phosphorylation of proteins associated with caveolae (Parton, 1994; Smart et al. 1995) or caveolin isoforms themselves (Aoki et al. 1999) stimulate caveolae to pinch off, and dephosphorylation of these protein might be required for recycling of these vesicles to the cell surface (Smart et al. 1995). The precise mechanism, substrates as well as kinases and phophatases taking part in this dephosphorylation procedure are not known. In order to study the effect of phosphorylation/dephosphorylation on the surface distribution and cytoplasmic localization of caveolin isoforms we have used ocadaic acid (OA). OA is a well-known serine/threonine (PP1 and PP2) protein phosphatase inhibitor (Wera and Hemmings 1995).

Our immunocytochemical studies showed that as a result of OA treatment in HepG2 cells the cytoplasmic and plasma membrane distributions of caveolin-1 and caveolin-2 have changed. Caveolin-1 has disappeared from the cell surface and was detected in vesicles arranged in rows in the cyto-

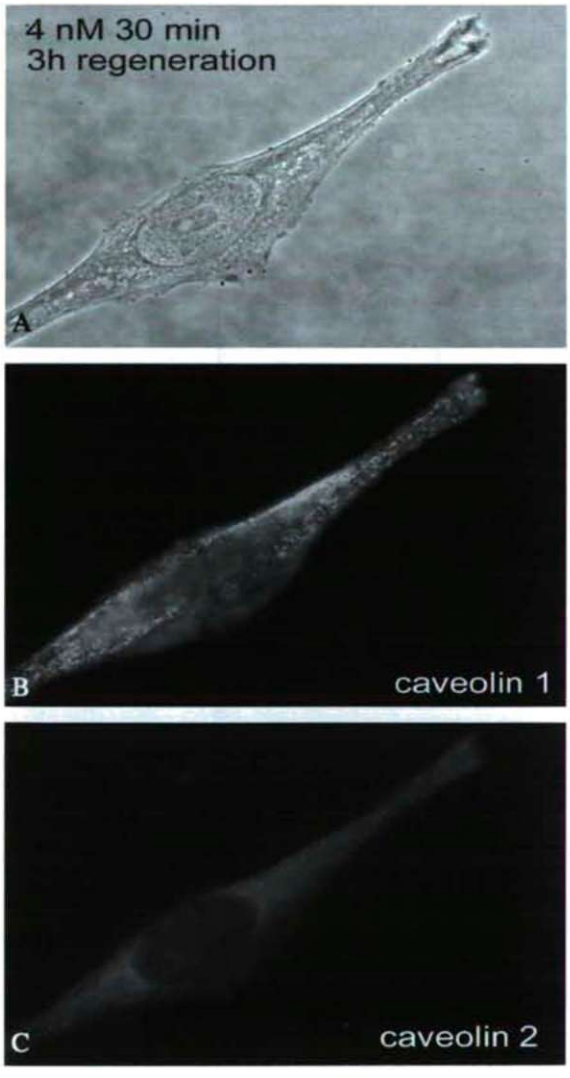

Figure 2. The effect of 4 nM OA treatment on the cellular distribution of caveolin-1 and caveolin-2. (**A**) phase contrast micrograph of HepG2 cells. After 30min OA treatment caveolin-1 has disappeared from the plasma membrane and was present in rows (**B**). Caveolin-2 was found in similar localization, but caveolin-2 containing perinuclear ring was still present (**C**). Magnification: 1000x

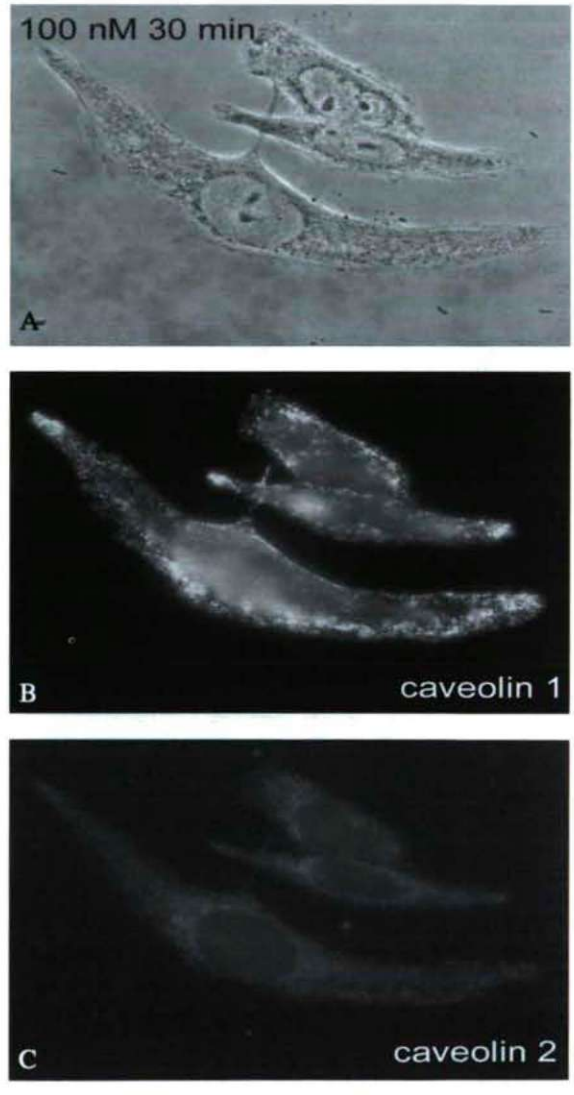

Figure 3. The effect of 100 nM OA on the cellular distribution of caveolin-1 and caveolin-2. (**A**) phase contrast micrograph of HepG2. Caveolin-1 was present in the periphery of the cytoplasm and it was arranged in rows (**B**). The caveolin-2 localization was similar to the 4 nM OA-treated cells (**C**). Magnification: 1000x

plasm. Since caveolae can be characterized by the presence of caveolin-1 and caveolin-2 (caveolin-2 seems to play an accessory role in caveolae-formation), the cytoplasmic route of caveolae can be followed by detection (localization) of these isoforms. From these data we think that caveolae have also disappeared from the cell surface. Although caveolin-2 was partly colocalized with caveolin-1, there was a significant amount of this isoform present in the perinuclear region. When OA was removed and the cells were incubated with an OA-free medium for 3 hours the localization pattern of the caveolin isoforms has changed again. Caveolin-1 was still arranged along cables, while caveolin-2 was detected around the nucleus. This perinuclear caveolin-2-containing ring was more prominent then in control cells. We were not able to detect caveolin-1 and –2 in punctate structures on the cell membrane. Our immunoprecipitation and Western blot analysis showed that OA treatment has resulted in tyrosine phosphorylation of a ~24 kDa protein which was identified as caveolin-2. When the cells were incubated with OA-free medium for 3 hours the tyrosine phosphorylation of the

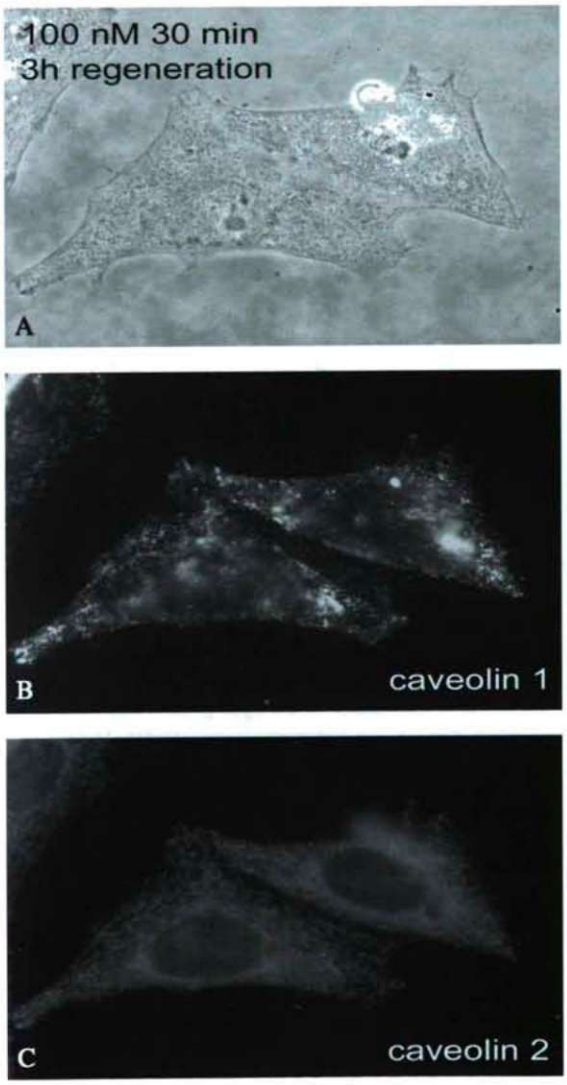

Figure 4. When 4 nM OA was removed and the cells were incubated in OA-free medium for 3 hours caveolin-1 was still present in the cytoplasm (in rows) and not on the cell membrane (**B**). The distribution of caveolin-2 has not changed (**C**) Magnification: 1000x

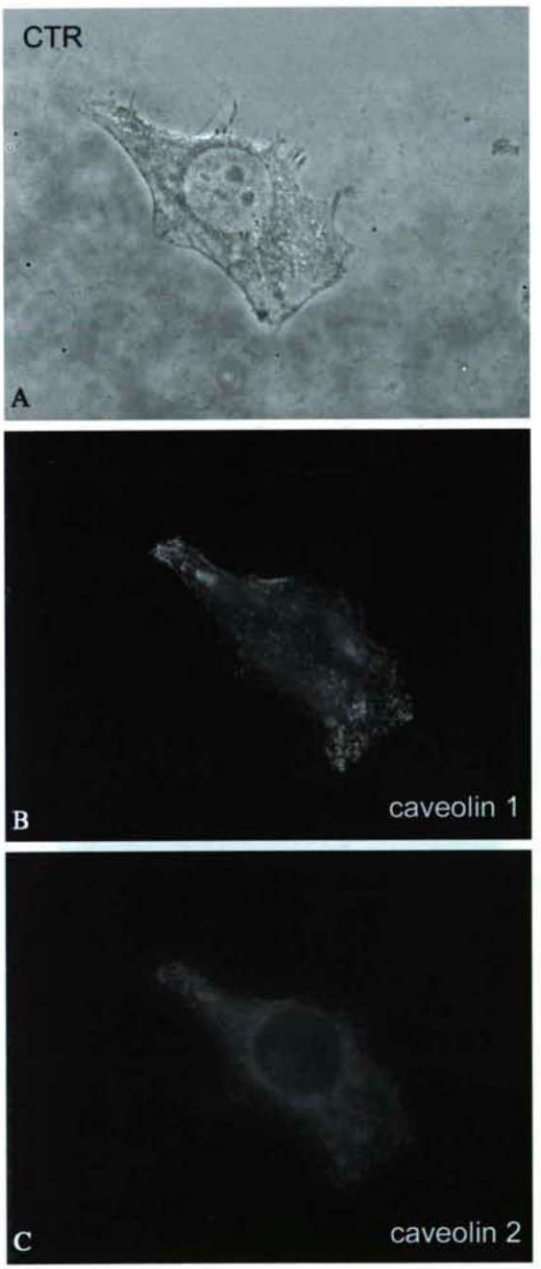

Figure 5. After 3 hours incubation in OA-free medium the effect of 100 nM OA was not found to be reversible. There was no caveolin-1 containing vesicles present on the plasma membrane (**B**), and caveolin-2 was found around the nucleus (**C**). Magnification: 1000x

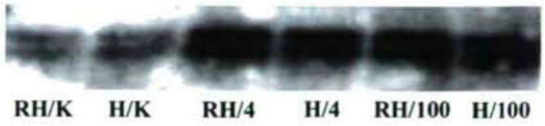

Figure 6. Immunoprecipitation with anti-caveolin-2 and Western blot analysis with anti-phosphotyrosine antibodies. Caveolin-2 (24 kDa) was strongly labelled on tyrosine residue when the cells were incubated with 4 and 100 nM ocadaic acid. 3 hours incubation without OA has not resulted in the dephosphorylation of caveolin-2. (RH/K: Control HepG2 cells 3 h in culture medium; H/K: control HepG2 cells 30min in culture medium; RH/4: HepG2 cells treated with 4 nM OA and 3 h in OA-free medium; H/4: HepG2 cells treated with 4 nM OA; RH/100: HepG2 cells treated with 100 nM OA and 3 h in OA-free medium; H/100: HepG2 cells treated with 100 nM OA.)

caveolin-2 became stronger indicating that the effect of OA was not reversible. Although OA is a serine/threonine phophatase inhibitor, we propose that OA treatment causes tyrosine phosphorylation of caveolin-2 indirectly, through phosphorylation of other members of a phosphorylation cascade. It is known that the Src family tyrosine kinases can be efficiently blocked by PP1 and PP2 phosphatases (Marinissen and Gutkind 2001). Since caveolin was first identified as a substrate for phosphorylation by v-src (Glenney and Zokas 1989), and Src kinases were also found to be associated with caveolae (reviewed by Anderson 1998), we suggest that OA causes tyrosine phos-phorylation of caveolin isoforms through this phosphorylation cascade.

From these results we conclude that the tyrosine phosphorylation of caveolin-2 can be responsible for removal of caveolae from the cell surface. Since there are data suggesting that dephosphorylation of proteins associated with caveolae would be necessary for caveolae recycling (Smart et al. 1995), we think that in OA treated cells caveolae can not recycle back to the cell surface. Our data strongly suggest that tyrosine phosphorylation of caveolin-2 plays an important role in the regulation of caveolin and caveolae distribution/localization in the cytoplasm. Cytoskeleton seems to be involved in this procedure.

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ARTICLE

Structural and functional changes of cell junctions on effect of ionizing radiation

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ABSTRACT Cell junctions are specialized regions of the cell membrane that are responsible for the formation of physical connections among the cells. They are also playing a role in cell communication and in the signal transduction processes of the plasma membrane. Following ionizing irradiations, their structural as well as functional changes are clearly detectable. They are generally able to modify the overall cell responses to various agents, whereas in some cases their functional changes can also be brought into relation with some end-points, such as apoptosis or tumor formation. According to experimental data, biologically active molecules (free radicals, signal transfer molecules) formed on the effect of radiation can get across into neighboring cells through the gap junctions that are responsible for intercellular chemical communication, and may there bring about changes characteristic to radiation injury (bystander effect) and they are also involved in/contribute to the so-called radio-adaptation of the cells. The permeability changes in the tight junctions caused by irradiation can be detected by EM and also by biochemical methods. Using morphometric analysis of EM specimens made by freeze-fracture technique, or by immunohistochemical detection of proteins derived specifically from tight junctions, it can be established that there are structural modifications occurring due to irradiation by X-rays. Using tissue culture model systems, radiation induced redistribution of cadherin and ß-catenin, two characteristic structural proteins of adherent junctions, is also detectable. Other molecules are also affected. Their expression or changes could play an important role in the development of acute or delayed injuries, including inflammatory processes occurring in the tissue - either as a cause or as an effect.

KEY WORDS

irradiation
gap junction
tight junction
adherens junction
radiation injury

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Animal cells in tissue organization are establishing and maintaining connections with each other as well as with the intercellular matrix. The build-up scheme thereof is a rather simple one. They possess one or more coupling protein(s) embedded in the plasma membrane, bearing extracellular side chains, which are linked to proteins of the neighboring cells or of intercellular matrix having the same function or structure. These proteins are also linked to a membrane-integrated protein, whose side chain is building up connections to cytoskeletal elements at the inner (cytoplasmatic) side of the cell membrane. From chemical point of view, these proteins establishing direct links between cells or cell-to-matrix connections can be of various kinds (Hynes 1999; Lodish et al. 2000). In tight junctions (TJs) this linking function is provided by JAM, occludin and claudins (Mitic and Anderson 1998; Stevenson and Keon 1998) while in the adherens type junctions the same function is attributed to cadherins (Takeichi 2000). In addition, such coupling proteins may originate from the family of immunoglobulins, some mucine-like proteins, integrins, lectins (p-selectin) (Hynes 1999; Lodish et al. 2000; Gottardi and Gumbiner 2001).

The coupling proteins that are establishing links to cytoskeleton are also dependent on the type of cell junction; these are the ZO proteins in case of tight junctions, while at adherent junctions the same role is played by the α- and β-catenins (Mitic and Anderson 1998; Hynes 1999). The various types of cell junctions regularly form cell coupling complexes in the epithelial cells by ordering themselves in a particular array matrix (Lodish et al. 2000).

The cell contacts, for example the tight junction or some adherent junctions, possess a variety of functions. They play a role in developing and maintaining the mechanical stability of the tissues, are regulating the paracellular transport of molecules, and are fundamental in the development and maintenance of the polarity of the plasma membrane (Nusrat et al. 2000). Their change, *i.e.*, development, modification, or even cease of linkages in which they are involved, will provide a distinct signal to the cell which may – through various kinds of signal transduction mechanisms – then affect a number of cell functions (growth, development, protein

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synthesis, mitosis (Stevenson and Keon1998; Christofori and Semb 1999; Lodish et al. 2000; Tsukita and Furuse 2002).

The formation and change of cell junctions is fundamentally significant in tissue development, and is frequently related to their function. Their alterations may either initiate pathological processes or can play a pathophysiological role in their course thereof (Albelda and Buck 1990; Karczewski and Groot 1999; Nusrat et al. 2000).

Co-ordination of cell couplings (maintaining their structure and function) is realized via difficult, multi-level regulation systems, the particular details of which are not yet exactly known. Structural and functional alterations are controlled by regulatory processes representing at least three different levels of biological organizations, *i.e.*, physiological, cellular and molecular (Denker and Nigam 1998; Matter and Balda 2003). Regulation at the physiological level manifests itself as a joint resulting effect of many hormones, neurotransmitters, cytokines and growth factors. At cellular and molecular levels, tight junctions are regulated by different important signal transduction systems, such as phospholipase C, protein kinases, tyrosin kinase, phosphatase, Rho-GTP-ase. Signal transfer molecules (secondary messengers; cAMP, cGMP and NO) seem to play decisive role in this regulation both in coordinating as well as in subordinating relations.

As far as the structure and function of cell couplings are regarded, some other regulatory options also exist, namely: directly through the change of the internal relationship among cell coupling proteins, via modification of the intensity of molecular connections within the junctional complex, by the enzymatic degradation of structural proteins, and by changing the strength and the extent of the links attaching them to the cytoskeleton. Indirect regulation is presumably occurring via the change of the half-lives of some constituting proteins of the given cell coupling, or by alterations in the transcription or synthesis thereof.

Structural and functional changes of cell couplings following ionizing radiation exposures

Following exposures to ionizing radiation a number of reversible and irreversible responses on cellular, tissue, and organ levels can be detected. There is a sequence of events spread in space and time and the resulting physical, chemical, and biological changes effected by the absorbed radiation energy that can be observed. Should the energy absorption occur at different macromolecules of vital importance, this may then cause a direct break in the DNA molecule (Lett 1992). Breaks to macromolecules may also be affected indirectly via the mediatory effects of radiolytic products of water and of several reactive free radicals (Livesey et al. 1985). Impairments of the macromolecules due to direct and indirect effects may lead to cell death, to an irreversible impairment of the genetic matter, including tumorous transformation and to a number of other pathologic changes, *e.g.* giving rise to an inflammation. Pursuant to experimental data available the change in cell couplings is in direct or indirect way – often involved in developing radiation injuries. Based on some observations, the communication via gap junctions, or eventually a particular change in it after irradiation, is influencing some end-points (tumorous transformation, cell death) of the radiation effect (Trosko and Inoue 1997; Wilson et al. 2000). The radiation impairment of the tight junctions and the epithelial junctional complex may play role in the pathogenesis of inflammatory processes.

The role of gap junctions in developing radiation injuries / impairments

The gap junctions can be regarded as the organelles of intercellular chemical communication. Its coupling proteins, the connexins, are forming channels between the cells, through which different ions and small molecules get across under regulated conditions (Lodish et al. 2000; Skerret 2002). Irradiation induces the connexin expression in the skin of mice (Liu et al. 1997) and in alveolar cells (Kasper and Traub 1996).

The radiation susceptibility of cells grown in suspension or in two-dimensional cultures has proved to be greater than that of spheroids or tissues made up from the same cells. (Durand and Sutherland 1972; Dertinger et al. 1993; Green et al. 2002). In general, it can be stated that the presence of gap junctions, the increase of the amount of their proteins is accompanied by the lowering of radiation susceptibility of the cells (Lin et al. 2003). These experimental data, suggest that the expression of connexin, beyond its radioprotective effect, has a certain protective role of rather general character against different harmful effects. The metabolic coupling and metabolic cooperation bound to cell couplings may result in both radio-protective as well as radio-sensitizing effects. Thus the gap junctional communication has a proven role in the radio adaptation of cells (Ojima et al. 2001). The role/ contribution of gap junctions is also significant in the observed so-called bystander effect within the domain of biological effects of ionizing radiation (Azzam et al. 2001; Little et al. 2002; Lorimore and Wright 2003). The biologically active molecules (free radicals, signal transmitter molecules, etc.) produced in the respective cells on effect of radiation get across via the gap junctions to the neighboring cells, directly not hit by radiation injury, where they can cause death or impairment of that cells, too.

Functional and structural changes of tight junctions following ionizing radiation exposures

The tight junction is a sophisticated molecular complex establishing multifunctional connections among epithelial

cells. This cell coupling stabilizes the neighbouring cells, regulates the intercellular transport of ions and of paracellular matter, inhibits lateral shift of membrane proteins; its signal transmitter and signal generating function is also well known (Mitic and Anderson 1998; Stevenson, and Keon 1998).

In tissues covered by different epithelial cells (kidney, small intestine, brain capillaries, etc.), the radiation injuries of the cell couplings play an important role-either as a cause or as an effect-in the pathogenesis of acute or delayed pathologic processes (alterations in permeability barriers, inflammatory processes) occurring in the tissue on effect of radiation (Robbins and Bonsib 1995; Somosy 2000; Dör and Hendry 2001; Robbins et al. 2002; Somosy et al. 2003, 2002).

The early increase of permeability of the intestinal wall and the subsequent progression of it, associated with inflammation, is well known. This functional change is the basis of evolution of the gastrointestinal syndrome on effect of irradiations with high-dose which may cause death of the organism (Young 1987). Permeability changes and accompanying acute and chronic pathologic processes can be detected also in the case of smaller, therapeutic dosages. Thus, applying ruthenium-red technique, it has been demonstrated in mice that following application of relatively low doses (3, 5 Gy) during whole-body irradiation experiments, presence of the tracer ruthenium-red, characteristic to the enhanced paracellular permeability can be detected among the epithelial cells covering the intestinal mucosa (Somosy et al. 1993).

Parallel to the functional changes there are data available from direct electron microscopic and immunohistochemical investigations, that point to the structural damages of cell couplings due to irradiation. Morphometric analysis of freeze-fracture specimens from small intestine epithelial cells of X-ray irradiated mice reveals that irradiation causes a reversible loosening of the molecular structure of tight junctions (Porvaznik 1979; Somosy 2000; Páfia et al. 2001; Somosy et al. 2002). By immunohistochemical investigation of distribution of occludin, one of the main structural proteins of the tight junctions in MDCK- and in HT-29 cell lines, we demonstrated the dose- and time-dependent nature of changes brought about by X-ray irradiation (Somosy et al. 2002; Somosy et al. 2003). In unexposed MDCK cells occludin was detected in a circumcellular distribution at the cell periphery and showed characteristic honeycomb-like pattern indicating the tight junctional zone (Fig 1). The x-ray caused a time- and dose-dependent reduction in the staining intensity of occludin as well as breaks in the mainly continuous lines of stain (Fig. 1). The extent and distribution of staining of occludin are dependent on its structural organization and on the barrier function (Balda and Matter 1998).

As it was mentioned earlier cAMP and cGMP have an important contribution to the regulation of permeability of endothelial cells and to the determinination of the structural integrity of cell couplings (Dye et al. 2001; Wan et al. 2001). A high level of cAMP stabilizes the structure of the cell couplings, diminishes the permeability, while an elevated level of cGMP impairs the structure of the cell couplings, and an increases the paracellular permeability. These data indicate that the equlibrium of the local concentrations of these signal transmitters, and the regulatory processes influenced by them, determine the maintenance of the structural and functional integrity of cell couplings. According to our recent data (Somosy et al. 2003), treatment with dibutyryl-cAMP (db-cAMP), increases the cAMP level, and the disorganization of the tight junctions caused by X-ray irradiation can be prevented. At the same time, no considerable irradiation damage of tight junctions was observed, by inhibiting the enzymes responsible for the production of NO, the NO-synthases (NOSs), either. To the contrary, a treatment with db-cGMP, known to increase the cGMP level, was able in itself to induce such damages similar to those brought about by irradiation Based on these experimental results, it can reasonably be supposed that the NO-cGMP-cAMP-system might be a regulating element of essential significance in the development of permeability changes following exposures to ionizing radiations.

It is to be noted that the role of the NO-mediated system plays in the pathomechanism of inflammations following irradiation appears to be significant, and is thus consequently important in the permeability increase associated with the late occurring inflammation.

The permeability increase within the therapeutic dose range of the permeability barrier found in the brain capillaries, *i.e.* of the blood-brain-barrier (BBB), due to irradiation is also known (van Vulpen and al. 2002; Yuan et al. 2003). The same phenomenon has been described in the literature (Diserbo et al. 2002) after a smaller, single dose in whole-body irradiation experiments, too. This phenomenon is also correlated with the radiation impairment of the endothelial cells covering the surfaces of the brain capillaries. The regulation of permeability of the BBB is a complex, multi-level system. There are no experimental data available in details regarding the possible mechanism of the radiation effect. It should be noted that the permeability increase of the BBB following irradiation is being employed in the combined therapy of certain brain tumours.

Changes of adherens type junctions following irradiation

The adherens type junctions constitute a considerable group of cell couplings in which the binding protein is cadherin, whereas the catenins are responsible for establishing cytoskeletal links. They participate in the formation of the cell coupling complex of epithelial and of endothelial cells. Two different cell couplings of the complex, namely the adherens

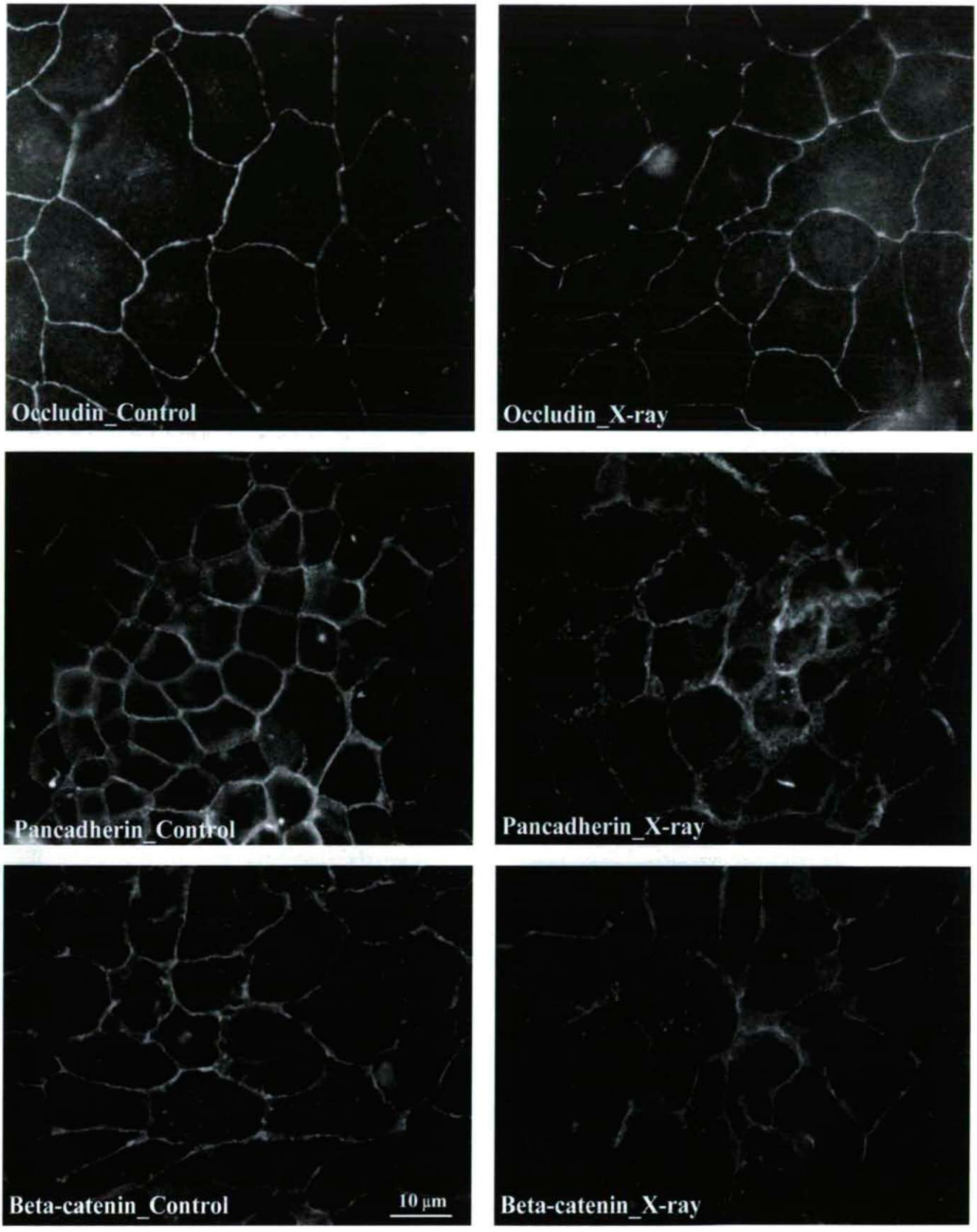

Figure 1. Binding characteristics of anti-occludin, pancatherin and beta-catenin antibodies to Madin-Darby Kidney cells.

junctions and the desmosomes belong to this group (Hynes 1999). In the cell coupling complex they are arranged behind the tight junction to which they are functionally and structurally closely connected. There are evidences concerning their involvement in determining the extent of permeability, and in addition, in the signal transmission processes. Adherens type junctions are essential in cell-to-basement links, too. In this case the protein embedded in the membrane, belonging to the cadherin group, is attached by heterofolic adhesion to some particular proteins of the matrix, like integrin and fibronectin; the cytoskeletal linker proteins could be vinculin, tropomyosin, and α-actinin. (Gottardi and Gumbiner 2000; Lodish et al. 2000; Blaschuk and Rowlands 2002). These connections are fundamental in determining cell shape and in the maintenance/stabilization of it, as well as the moving of cells (Hynes 1999; Lodish et al. 2000).

Irradiation and the adherens junctions in junctional complex

On effect of ionizing radiation (^{60}Co γ-radiation, whole-body) a quick, reversible loss of cadherins (E and OB) was found in rat colon crypt cells and in pericryptal myofibroblasts as well (Thiagarajah et al. 2000). This loss is also coincides with the enhanced permeability of the colon mucosa, which allows to conclude to a direct, cause-and-effect type link between the two phenomena. The amount of β-catenin, being the cytoskeleton linker protein of cadherin, also decreases following irradiation (Fig. 1). Our experiments carried out *in vitro* on MDCK-cells have led to similar result. We used pan-cadherin antibody which was able to detect cadherins both in basal and in lateral positions. X-ray irradiation affected the lateral marking giving rise to its significant decrease, while the basal marking did not change in an appreciable manner. We have also experienced the loss of β-catenin in our experiments.

The reason for the detected structural changes could not yet be elucidated, and the eventually accompanying, irradiation-caused functional modifications are not yet known in details. Pursuant to the view of Thiagarajah et al. (2000) an indirect process could be behind, which is triggered by caspase 3, having been induced by the irradiation. According to their hypothesis the observed loss of E-cadherin in crypts is in correlation with that of the water addsorption of the colon. In addition, the observed changes in the pericryptal myofibroblasts could give an explanation to the disintegration of the pericryptal sheet(s).

Hardy et al. (2002) showed that a considerable expression of P-cadherin, a kind of cadherins normally not present in colon, occurs parallel to translocation of E-cadherin and catenin associated thereto, from the membrane. Pursuant to their view, this cell coupling protein, *de novo* synthetized in that particular tissue, may promote/facilitate the regeneration of the tissue in a way. On the other hand many researchers (Akimoto et al. 1998; Ebara et al.1998) working on tumour cell lines (lung carcinoma, A549 and thyroid gland carcinoma T-SCC) have observed a time- and dose-dependent increase of E-cadherin and of α-catenin, and have described a fall in cell migration.

Changes in the cell-matrix and cell-substrate connections

Irradiation could change cell shape. This phenomenon was observed on irradiated primary human fibroblasts (Somosy 2000), lung artery endothelial cell cultures (Friedman et al. 1986) and neuroblastoma cells. The ruffling activity on fibroblasts and on neuroblastoma cells increases and characteristic to the moving cell forms. The stuck cell surfaces are partly splitting off, ruffles, microspikes and blebs are going to occur (Hamberg et al. 1978; Somosy 2000). Ionizing radiation modifies the expression of various integrines on cell surface (Meineke et al. 2002), as the cell-matrix connections may be subject of modification. In some cases an increase in cell migration has been found (Wick et al. 2002) while in others the irradiation brings about an enhancement of adhesion (Cordes et al. 2002). It is also known that irradiation modifies the composition of the extracellular matrix. Giannopoulou et al. (2001) reports that 6 hours following an exposure to X-ray the irradiation has diminished both the amount and the expression of the relevant genes of fibronectin and laminin. Changes in cell-matrix connections after irradiation are dependent of the alterations in the binding proteins, both on the cell surface and in the matrix as well.

Quantitative and distribution changes of adhesion molecules that play role in the pathomechanism of inflammation development following irradiation

Ionizing radiation is known to evoke both acute and chronic inflammatory reactions in several organs and tissues, such as skin, intestinal system, lungs and kidneys. The development of inflammatory response is a finely regulated process that involves sequential lecocyte-endothelial or epithelial interactions designed as rolling, activation, adhesion, and emigration. By changing the connections between these two cell types, in relation to this phenomenon, changes in the expression of various cell coupling molecules, such as selectines, integrins, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) can be observed (William 2002).

After giving higher doses (2 to 20 Gy) in a number of *in vivo* and *in vitro* experimental setups of endothelial cell systems, dose- and time-determined quantitative changes of the E-selectin, P-selectin, ICAM-1, VCAM-1 have been described (Hallahan and Virudachalam 1999, 1997; Molla et al. 2001). According to these data the up-regulation of these

adhesion molecules is in correlation with the enhanced concentration of various intercellular mediators occurring upon effect of radiation (Barcellos-Hoff 1998; Gorbunov et al. 2000; Köteles és Somosy 2001). It is also probable that hydroxi radicals, appearing as an early effect of radiation, have an essential role in the development and course of inflammation diseases. NO is involved in the pathomechanism of the development of inflammations (Freeman and MacNaughton 2000; Leach et al. 2002).

It is of considerable interest that low-dose irradiations (0.3-0.7 Gy) have an anti-inflammatory effect, contrary to that of higher doses. Hypothetically, it may be assumed that in the course of development of this antiphlogistic effect, these doses are diminishing the expression of L-selectin that is fundamental in the initial steps of the inflammatory process (Roedal et al. 2002).

Conclusions and Perspectives

It is evident from the brief summary above that structural and functional alterations of cell couplings are of essential significance from the point of view of development of changes following the radiation-organ and radiation-tissue interactions. Thus the early change in permeability of the endothelium or of the epithelium covering different hollow organs, the inflammatory processes, and the accumulation of connective tissue appearing as a late effect, can play a role in the alterations of the cell couplings both as a cause and as an effect. Changes in cell-to-cell communication occurring on effect of radiation, in the evolution of which many types of cell couplings (gap junction, adherens junction, integrin mediated connections) are involved, are of significance also in development of both the early and the late effects of radiation. From these important roles of cell couplings it is to be concluded that those regulating processes, and those biologically active compounds which influence the alterations of the cell couplings, contribute to the modification of the effect of radiation, too, and thus it is conceivable that they can have a role in radioprotection or in enhancing the effectivity of radiotherapy by making the cells more sensitive to radiation. It is therefore comprehensible that the antiphlogistic treatment based on the modification of connections of the cells is effective in radiotherapy, or the NO inhibitors having a stabilizing effect on the tight junctions, possess a radioprotective effect. It can be predicted that results of these research will help in preventing radiation injuries/damages associated with irradiations of therapeutic purpose or those deriving from accidental irradiation exposures.

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ARTICLE

Effect of 50 Hz magnetic field exposure on the adherent cell contacts of primary mouse Leydig cells in culture

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ABSTRACT Mouse primary testicular intersitital (Leydig) cells obtained from 30-35 g NMRI mice were exposed in a CO_2 incubator to a sinusoidal 50 Hz/100 μT magnetic field for 48 h. Non-exposed, human choriogonin stimulated (0.1 mIU/ml hCG) cells were also cultured as positive controls. Cells were grown as monolayer on cover slips posited on the bottom of the plastic 24-well culture plates. Following the incubation the cells were fixed and permeabilized with -20°C methanol for 2 hours. For immunocytochemical detection of cadherins, ß-catenin and tubulin, cells were incubated over 60 minutes at room temperature with (1:300 diluted) pan-cadherin, anti-ß-catenin or antitubulin. Anti-mouse FITC developed in rabbit was used as secondary antibody. Evaluating the samples by fluorescent microscopy, we found that the applied magnetic field exposure increased the amounts of cadherins and ß-catenin along the surface of the cell-to-cell contacts. The amount of microtubuli was also elevated and typical shape of cells was changed. The effects of magnetic field exposure were similar to those caused by hCG in the positive controls. Further investigations are required to clarify the subcellular action of applied magnetic field in Leydig cells. **Acta Biol Szeged 47(1-4):27-30 (2003)**

KEY WORDS

magnetic field
cadherin
catenin
tubulin
immunocytochemistry
Leydig cell

With the increasing distribution and utilization of electrical machines and electronic equipment in our society, concern about the possible effect of the electromagnetic fields on our environment on our health is a topic of increasing interest. One of the most important field of research in this topic the investigation of the possible biological effects of power-line frequency (50/60 Hz) magnetic fields.

In our previous experiments we found that in vitro exposure to sinusoidal 50 Hz 100 μT magnetic fields was able to stimulate the testosterone (T) production of mouse Leydig cells in a 48-h primary culture (Forgács et al. 1998a). Similar response was detected following an in vivo 50 Hz, 100 μT magnetic field exposure performed over a period of 14 days (Forgács et al. 2001). In the last decade, several investigators reported that Leydig cells also expressed cell-to-cell adhesion molecules such as cadherins and catenins (Byers et al. 1994; Denduchis et al. 1996).

Hence, the main goal of this study was to evaluate whether the magnetic field-induced functional changes of Leydig cells were accompanied by some morphological alterations or not. In the experiments reported here, we investigated the possible effect(s) of the in vitro 50 Hz magnetic field exposure on the adherent cell contacts and microtubular system of mouse Leydig cells in primary 48-h culture.

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Materials and Methods

Animals

NMRI mice (Charles River, Hungary) weighing 30 to 35 g (8-9 week old) were kept in a room with a 12:12 light/dark photoperiod, temperature of 20-23 °C, and relative humidity of 50-60%. Animals were housed eight per cage and allowed free access to standard laboratory pellets and tap water. In each experiment 20 mice were anesthetized with 60 mg/kg ip. pentobarbital (Rhone-Poulenc Rorer, Vitry sur Seine, France) and testes were removed. After surgery, the animals were overdosed with pentobarbital. The mice were kept and handled according to the guidelines of the Hungarian Law of Animal Care.

Interstitial cell preparation and incubation

For isolation of interstitial (Leydig) cells by mechanical dissociation without enzyme treatment the method of Stoklosowa (1982) was used with some modifications, as described earlier (Forgács et al. 1998b). Four independent experiments were performed. In each experiment the cells (obtained from testicles of 20 mice) were isolated in one

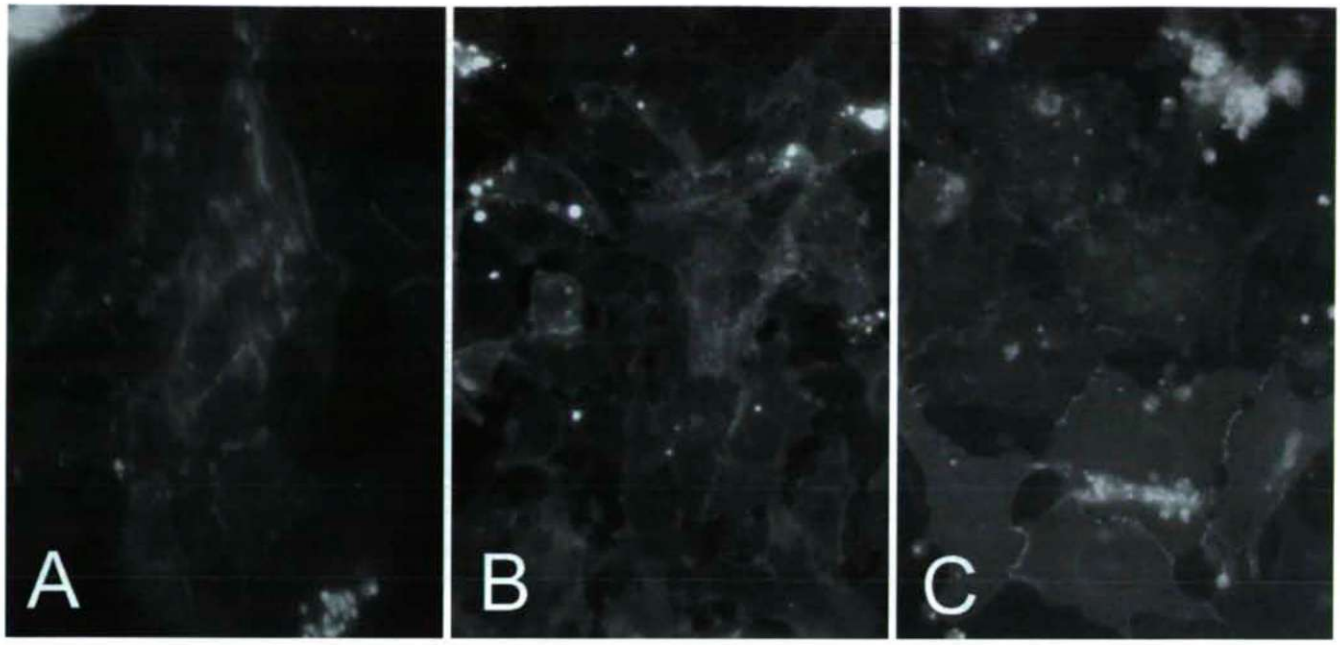

Figure 1. Localization of cadherins in 48-h mouse primary Leydig cell culture detected by pan-cadherin antibody. (A) Non-exposed (controls); (B) non-exposed + human choriogonin-stimulated (0.1 mIU/ml hCG; positive controls); (C) 50 Hz/100 μT magnetic field-exposed cells. Pan-cadherin antibody sdilution was 300:1. Photomicrograph was taken by a fluorescent light microscope (Zeiss Axioskope), with magnification of 400x.

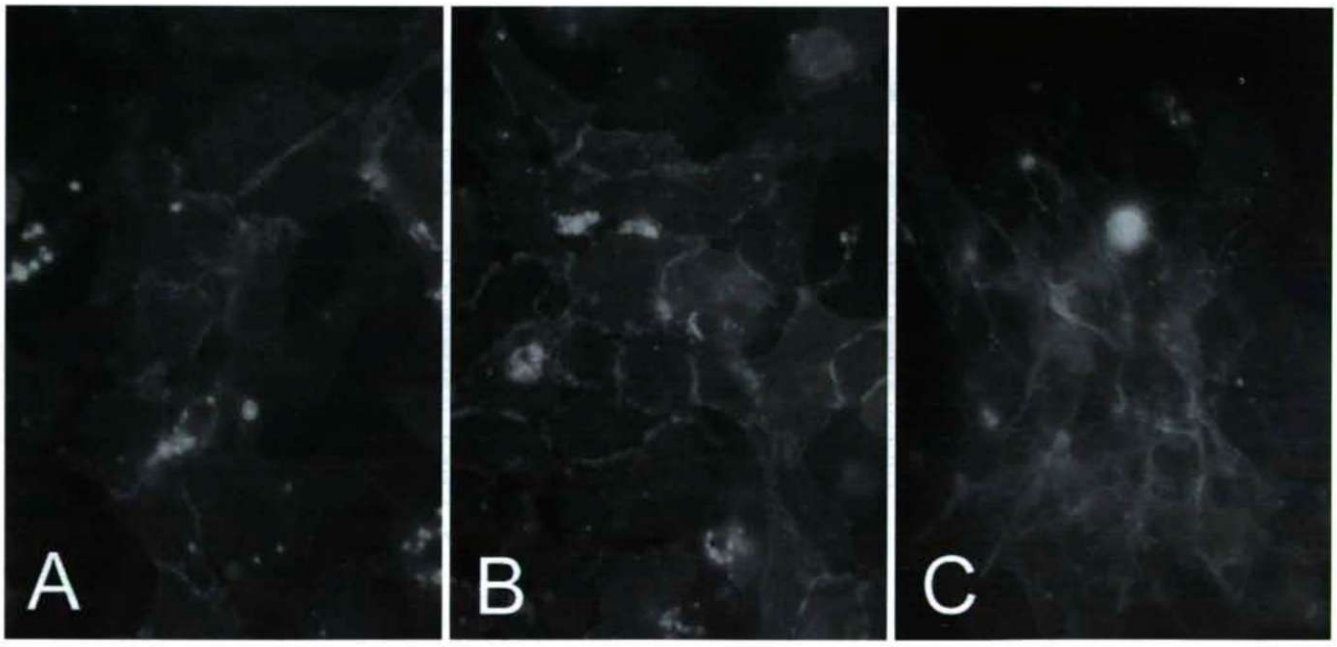

Figure 2. Localization of β-catenin in 48-h mouse primary Leydig cell culture detected by anti-β-catenin antibody. (A) Non-exposed (controls); (B) non-exposed + human choriogonin-stimulated (0.1 mIU/ml hCG; positive controls); (C) 50 Hz/100 μT magnetic field exposed cells. The anti-β-catenin antibody dilution was 300:1. Photomicrograph was taken by a fluorescent light microscope (Zeiss Axioskope), with magnification of 400x.

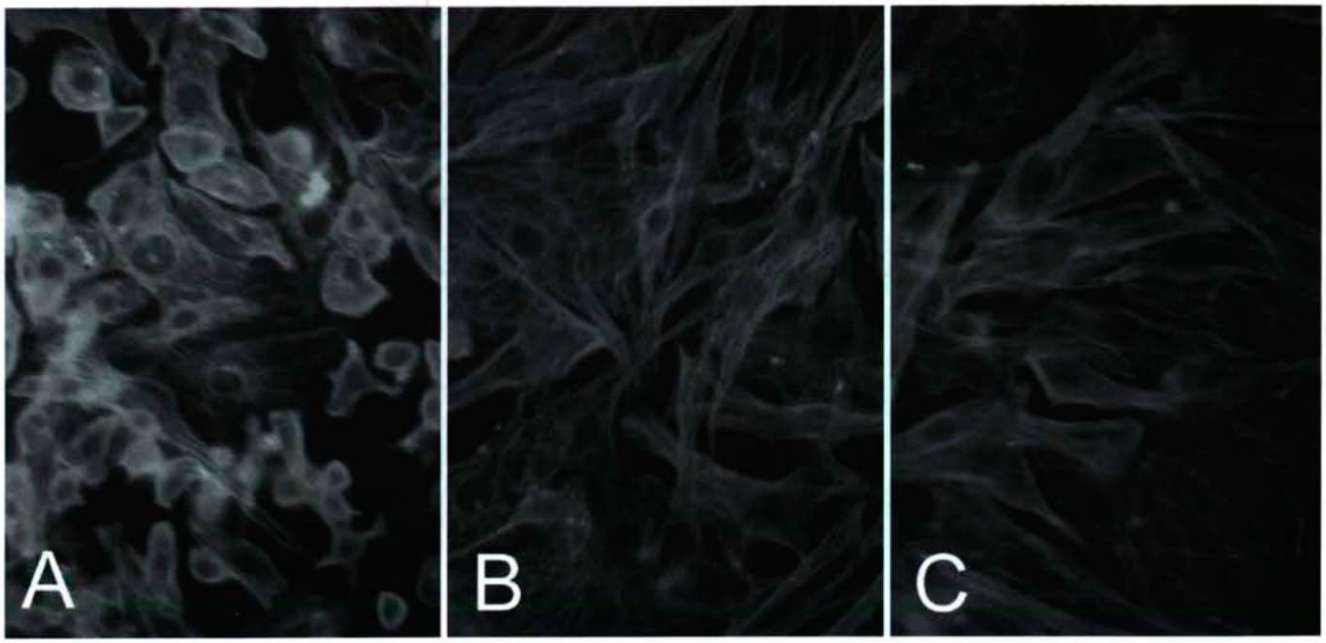

Figure 3. The microtubular structure of 48-h mouse primary Leydig cell culture detected by antitubulin antibody. (A) Non-exposed (controls); (B) non-exposed + human choriogonin stimulated (0.1 mIU/ml hCG; positive controls); (C) 50 Hz / 100 μT magnetic field exposed cells. The antitubulin antibody was purchased from Sigma, the dilution was 300:1. Photomicrograph was taken by a fluorescent light microscope (Zeiss Axioskope), with magnification of 400x.

block. The interstitial cell suspension was diluted to 10^6 cells/ml in (Sigma Chemical Co., St.Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma), 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma). The cell suspension was plated (with final volume of 500 μl/well) into sterile 24-well plates (Corning Glassworks, Corning, NY, USA). Cells were grown as monolayer on cover slips posited on the bottom of culture plates.

All incubations were carried out at 34°C under a humidified atmosphere of 95% air/5% CO_2. Non-exposed (controls), non-exposed + human choriogonin stimulated (0.1 mIU/ml hCG; positive controls) and magnetic field exposed cells were cultured.

Magnetic field exposure

The cell cultures were exposed in a CO_2 incubator to AC magnetic field vertically parallel to cylinder axis of a cylindrical recording chamber. Since the lenght of the 12.8 x 8.5 cm culture plates was larger than that of the diameter of coils, only the central 16 wells of the plates were used for cell culture. The frequency of AC magnetic field was 50 Hz with the flux density B(AC) = 100 μT (rms). The duration of exposure was 48 h. Two coils in Helmholtz arrangement were used to produce the vertical AC magnetic field. The inner diameter of coils was 12 cm, the number of turns was 85 for each coil, the diameter of copper wire was 0.3 mm. The coils were connected in a parallel mode (as a pair) with the resultant resistance of 4.2 Ohm (De Seze, 1994). The magnetic field was measured by three dimensional magnetic field meter (Fluxset 3C) and one dimensional Hall-probe connected to gaussmeter (Lakeshore Model 410) and Wandel & Goltermann (EFA-3) magnetic field measurement system (Vertessy 1994). The spectral component of the applied and background magnetic field was analyzed as well. The resultant earth's magnetic field was 46 μT at the National Institute of Chemical Safety (where experiments were conducted). The background 50 Hz magnetic field in the incubator was 40/300 nT (heating off/on).

The stray field of the applied 50 Hz magnetic field was 1-4 μT at the place of the unexposed controls, while the first (100 Hz) and second (150 Hz) harmonics were less than 8% and 3% respectively. The inhomogeneity of the applied 50 Hz magnetic field was better than ±5% inside the chamber area where the cell culture measurements were performed.

The AC magnetic field was generated by power audio-complex generator (Type TR 0157; HT, Budapest, Hungary).

Immunocytochemistry

Following the incubation the aliquots of the culture medium were removed and the wells of the culture plates were twice washed with phosphate buffer saline (PBS). After washing, for the immunohistochemical detection of cadherins, ß-catenin and tubulin, cells were fixed and permeabilized with methanol at -20 °C for 2 hours. Pan-cadherin, anti-ß-catenin

or anti-tubulin obtained from Sigma (St. Louis, MO) were used in a 1:300 dilution for 60 minutes at room temperature. The FITC-labeled anti-mouse secondary antibody developed in rabbit was obtained from Sigma. Cells were then mounted in Vectashield mounting medium (Vector Lab. Inst. Burgilame, CA) and examined in Axioskope (Zeiss, Germany) fluorescent microscope. Pictures were taken on color slides (Fujichrome 200 or 400 ASA) and scanned in by a slide scanner (Minolta, Dimage Scan Elite II) with 2,800 dpi optical resolution.

Results and Discussion

We found that the applied magnetic field exposure increased the amounts of cadherins (Fig 1C) and ß-catenin (Fig 2C) along the surface of the cell-to-cell contacts detected by immunocytochemical methods. The amount of microtubuli was also elevated (Fig 3C) and the typical shape of cells was changed (Fig 1C, Fig 2C, Fig 3C). The effects of magnetic field exposure were similar to those that caused by hCG, which was used as positive control (Fig 1B, Fig 2B, Fig 3B).

The exact mechanism of action of the applied magnetic field on Leydig cell-to-cell contacts and morphology cannot discerned from the present results. A possible mechanism of action may be associated with the alterations in cAMP content, and intercellular communication may be induced by the applied field. Schimmelpfeng et al. (1995) found increased cAMP content and gap junction-mediated intercellular communication after 5 min of exposure to 50 Hz, 2 mT magnetic field in monolayers of SV40-Swiss-3T3 mouse fibroblasts at intermediate cell density. It is possible that in our prevoius experiments the testosterone production was increased due to an elevated cAMP level. It is known that cAMP is one of the most important second messengers in LH receptor-mediated steroidogenesis by Leydig cells. On the other hand, according to literature data, elevated level of cAMP has a stabilizing effect on cell contacts and increases the number of microtubuli.

Further investigations are required to clarify the subcellular action of applied magnetic field in Leydig cells, as well as to establish the biological significance of this phenomenon.

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ARTICLE

Influence of arbuscular mycorrhiza and cadmium on the polyamine contents of Ri T-DNA transformed *Daucus carota L.* root cultures

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ABSTRACT Influence of arbuscular mycorrhizal (AM) colonisation on free polyamine (PA) contents and ratios was investigated in vitro by Ri T-DNA transformed root cultures (*Daucus carota L.* and *Glomus intraradices Schenck & Smith*) under Cd-treatment. Roots were soaked in $Cd(NO_3)_2$ solutions of $3x10^{-6}$ M and $5x10^{-5}$ M concentrations for 6 hours. Roots were strongly colonised and their Cd-content increased in case of the higher Cd-treatment only. In contrast to earlier results, Cd treatment reduced putrescine and spermidine contents of the non-mycorrhizal (NM) *Daucus carota* roots, whereas that of mycorrhizal (M) roots did not change. Spermine content showed a slight increase in all cases. NM roots always had higher PA ratios. AM symbiosis may have established a more equalised environment for the roots and so decreased the volume of physiological responses induced by stress conditions, which are normally reflected by strong changes in polyamine contents.
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KEY WORDS

arbuscular mycorrhiza
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putrescine
root culture
spermidine

Arbuscular mycorrhiza (AM) is the most ancient and widespread form of the mycorrhiza symbiosis. AM fungi normally stimulate phosphorus uptake, growth and photosynthesis of host plants; in return, host plants provide carbohydrates for their fungal partners (Smith and Read 1997).

Heavy metal stress resistance of the plant partners is often improved by colonisation, however, a general mechanism still has not been established. The uptake of heavy metals by AM colonised plants may be higher in high heavy metal containing soils (Gildon and Tinker 1983, Guo et al. 1996), but in other cases, AM colonisation reduced the heavy metal content of plant tissues (Schüepp et al. 1987; Díaz et al. 1996). Tonin and co-workers (2001) showed the accumulation of the toxic metal in non-toxic forms in the root. Due to the greater absorbing surface, the uptake of Cu, Zn and Cd were increased by the mycorrhiza from polluted soil, but the volume of the translocation to the shoot was reduced (Loth and Höfner 1995; Joner and Leyval 1997). According to Schüepp et al. (1987), under high soil heavy metal content, Zn and Cd uptake decreased in AM plants. However, in addition to the reduction of the Cd uptake, the absorption of the Zn was stimulated when heavy metal concentrations in soil were low. The effect of the AM is strongly dependent on the type and concentration of the certain heavy metal, the pH of the environment/medium and the growth conditions. A general mechanism cannot be defined even under the same conditions, since different fungal strains often have a mechanism specific to them (Weissenhorn et al. 1995).

The accumulation of the Cd in the fungal structures with large heavy metal binding capacity may act as a biological 'barrier' system (Joner and Leyval 2001). Turnau and co-workers (1993) showed in *Pteridium aquilinum* roots, that heavy metals (Cd and Ti) were sequestered in the vacuoles intercellularly and in the extramatrical hyphae.

It is still unclear to what extent the inherent stress resistance mechanisms of the host plants are influenced by the presence of mycorrhiza, which may partly explain the great diversity in the heavy metal stress responses of AM plants according to Rivera-Becerril et al. (2002). The overall improved heavy metal (Cd) resistance of AM plants was called the 'buffer-effect' of the mycorrhiza (Rivera-Becerril et al. 2002), but detailed characterisation of this effect is still to be made.

Change in the hormonal balance (including polyamines) is a frequent response of plant metabolism to the mycorrhizal colonisation influencing many physiological aspects including stress resistance (Smith and Read 1997). Furthermore, several environmental challenges like mineral nutrient deficiencies or atmospheric pollutants (*e.g.* Cd) have profound effects on plant polyamine (PA) metabolism (Bouchereau et al. 1999). Putrescine (Put) seems to be a stress signal molecule and its concentration is usually increased under stress conditions. Our knowledge is very limited about the role of PAs in plant-microbe symbioses (Walters 2000), but they may take part in the molecular

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signalling events between the symbiotic partners (El Ghachtouli et al. 1995). Their concentrations and ratio may reflect the demand for the AM symbiosis in P deficiency stress conditions (Parádi et al. 2003). Since they have a basic role in stress physiology, PAs may also have a role in the improved heavy metal stress resistance of AM plants.

Materials and Methods

Ri T-DNA transformed carrot (*Daucus carota L.*) roots were provided by Dr. Guilloume Bécard (Lab. de Mycol. Pole de Biotech. Veget., Auzeville, Castanet Tolosan, France). Mycorrhizal root cultures were colonised by Glomus intraradices Schenck & Smith. Roots were cultivated on a minimal (M) medium described by Bécard and Fortin (1988) solidified with 0.3% Phytagel® (Sigma-Aldrich, St. Louis, MO, USA). After subculturing, roots were grown for six weeks in the dark at room temperature.

During treatments, roots with the agar discs were transferred to 1 L beakers containing 100 ml $^1/_4$-strength Hoagland solution (Fodor et al. 1998). For Cd treatment, $Cd(NO_3)_2$ in $3x10^{-6}$ M (Cd-1) and $5x10^{-5}$ M (Cd-2) concentrations were used. One of the controls was measured immediately after opening the Petri dish (Co-1), while the other control was soaked for 6 hours on a shaker (120 rpm) with the Cd-treated roots (Co-2). After treatment, root fragments were thoroughly cleaned of agar remnants and randomly homogenised.

Mycorrhizal colonisation was quantified according to Phillips and Hayman (1970) and Trouvelot et al. (1986). Cd contents of roots were measured by an JY238 Ultrace ICP spectrometer (Jobin Yvon/Spex Division, Longjumeau, Cedex, France) in the ICP laboratory of the Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Sciences, Budapest, Hungary.

Quantities of free PAs were determined by thin-layer chro-matography (Rácz et al. 1996) and by a Jobin Yvon-Spex Fluoromax-2 spectrofluorometer (Instruments S.A., Jobin Yvon/Spex Division, Longjumeau, Cedex, France). Put content compared to the total of spermidine (Spd) and spermine (Spm) contents (Put/(Spd+Spm) ratio) was calculated.

SPSS 7.5 was used to perform two-way ANOVA on the effects and interaction of treatments. Means were compared between treatments by the Student's *t*-test.

Results

Colonisation

The colonisation parameters of the mycorrhizal (M) roots were relatively high and had similar values. The average colonisation frequency (F%), the intensity of the colonisation (M%) and the arbuscule content of the roots (a%) were 92%, 75% and 42%, respectively. As expected, colonisation was not found in the non-mycorrhizal (NM) root cultures.

Cd uptake

The amount of Cd entering the roots in Cd-1 treatment did not differ from the control value. However, despite the lack of deviation (there was only one parallel measurement), it can still be established that heavy metal content of roots was much greater in case of Cd-2 treatment (Table 1).

Polyamine content

The Cd treatments and the presence of mycorrhiza had significant effects on the contents of PAs in all cases (Fig. 1, Table 2). The Put content of the NM roots decreased under both Cd treatments compared to the control values, but the decline was not affected by the amount of Cd used (Fig. 1). Typically, Put contents of the NM roots were always higher than those of the M roots, and the effect of the mycorrhiza was highly significant (Table 2). The Put content of the M roots did not change substantially.

The Spd content also decreased in the NM roots when treated with Cd, irrespectively of the amount of the Cd in the media. The soaking must have played a role as well as the presence of the Cd, in the decreasing of the levels of Spd. Examining the Co-2 treatment, the effects of soaking and Cd treatments can be separated (Fig. 1). The Spd contents of the M roots changed slightly under the different treatments. They were lower without and higher with Cd treatment than those of the NM roots. This is confirmed by the fact that the interaction of mycorrhiza and Cd treatments was highly significant as regards Spd content (Table 2). It can be concluded, that the extent of changes in the Spd contents caused by the heavy metal treatment are dependent on the presence of mycorrhiza.

In both the M and the NM roots, a slight increase was observed in the Spm levels after Cd application (Fig. 1), which proved to be significant (Table 2).

Similarly to the differences in the Put content, the effect of the mycorrhiza on the Put/(Spd+Spm) ratios was also significant (Table 2.), in mycorrhizal roots these were lower in all cases (Fig. 1).

Discussion

Colonization and Cd uptake

According to di Toppi and Gabbrielli (1999), soils containing Cd concentrations above 1 μM can be considered as being strongly polluted. In this study, the applied treatments of 3 and 50 μM Cd are within the lower range of the strongly polluted category.

The potential heavy metal binding capacity of the media might have influenced the amount of Cd entering the root tissues, which may explain why the Cd content of roots under the lower Cd treatment was very similar to that of the control (Table 1). It can also be hypothesised based on previous results (Turnau et al. 1993) that Cd might have also been

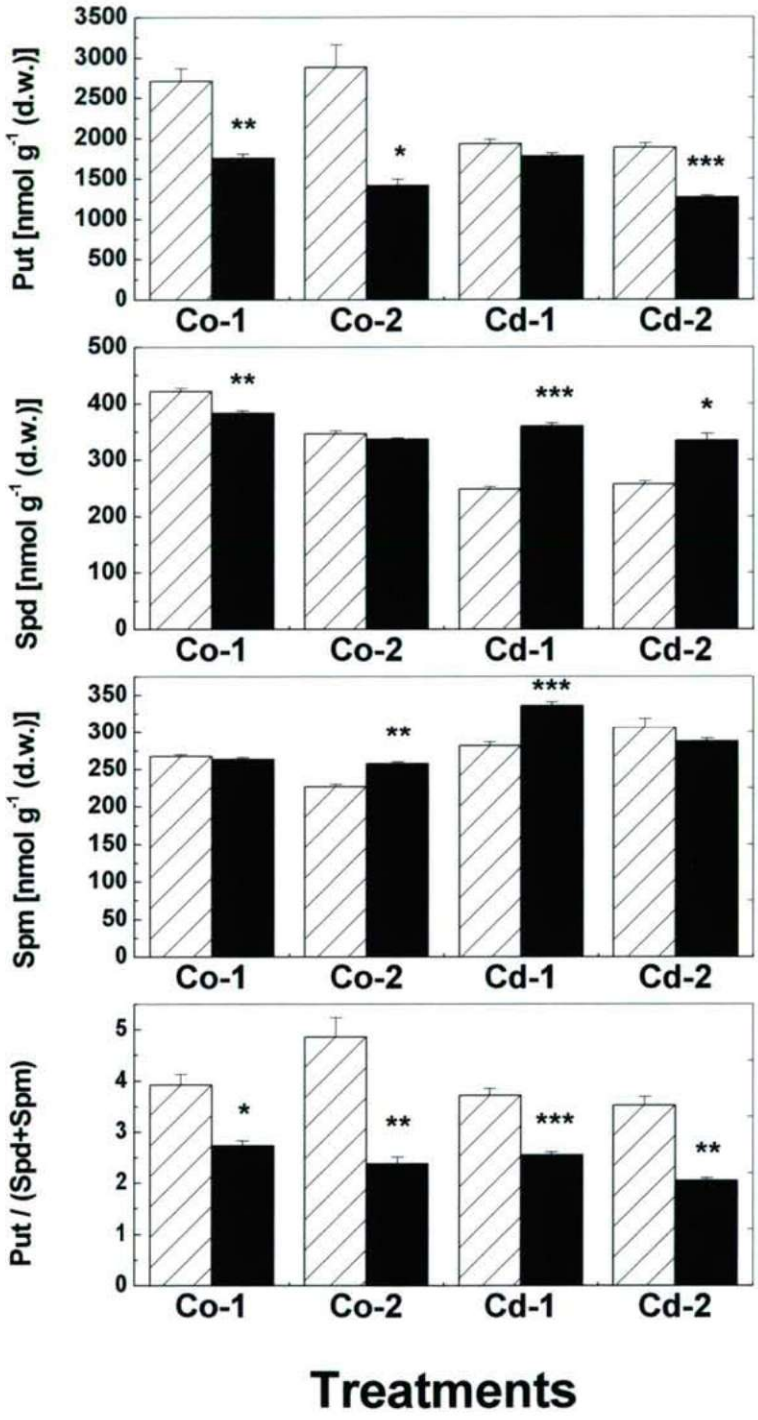

Figure 1. Average putrescine (Put), spermidine (Spd) and spermine (Spm) contents and polyamine ratios (Put/(Spd+Spm)) in mycorrhizal (M) and non-mycorrhizal (NM) Ri T-DNA transformed *Daucus carota* root cultures regarding to dry weight. SE is shown by vertical bars. Statistically significant differences between M (closed columns) and NM (hatched columns) plants under the same treatments are indicated as: *-P≤0,05, **-P≤0,01, ***-P≤0,001.

bound by the external hyphae of mycorrhizal fungi leading to lower Cd content of M roots in case of Cd-1 treatment.

The higher dose of Cd caused greater (1.5 times higher) accumulation in the M roots compared to the NM (Table 1). There is no information about the precise localisation of the Cd in the tissue, but it was postulated before (Joner and Leyval 1997) that substantial amount of Cd can be immobilised in the intra- or intercellular hyphae or structures of the mycorrhizal fungal partner. The toxic effect of Cd on the M root tissues could have been reduced by this immobilisation, in spite of the higher overall metal content. The accumulation of the Cd in a large heavy metal binding capacity fungal structure may act as a 'biological barrier' (Joner and Leyval 2001). The role of mycorrhizal fungi in adsorbing Cd can also be supported by the relatively high colonisation intensity in the present study.

Polyamine content

El Ghachtouli et al. (1995) were the first to show that PAs may have a role in the AM symbiosis, when colonisation frequency in peas was increased by applying PAs exogenously. El Ghachtouli et al. (1996) later proved that root growth and AM colonisation could be reduced by the application of PA biosynthesis inhibitors. This effect was reversed by the addition of exogenous Put. However, in the same experiment, no difference was found in the PA concentrations between mycorrhizal and control plants. Nevertheless, Goicoechea et al. (1998) found higher Spd and Spm concentrations in mycorrhizal alfalfa plants under water stress conditions.

It is well known that K deficiency has a considerable effect on the level of Put (Richards and Coleman 1970, Savonen and Sarjala 1998), and there is data on the possible role of the P deficiency (Parádi et al. 2003). In the present study, the roots were grown on a minimal media for the optimal growth and function of the mycorrhiza. This media contained low amounts of K and P (KH_2PO_4: 4.8 mg/l). Therefore, the higher Put levels and PA ratios observed in the NM roots (Fig. 1) can be also explained by the effect of the low K and P supply.

Table 1. Total Cd content of mycorrhizal (M) and non-mycorrhizal (NM) Ri T-DNA transformed *Daucus carota* root cultures.

Treatment	Cd concentration (mg/kg d.w.)
NM Co-1	0,33
M Co-1	1,00
NM Co-2	0,32
M Co-2	0,28
NM Cd-1	0,988
M Cd-1	0,492
NM Cd-2	106
M Cd-2	164

Treatments: without soaking and Cd (Co-1), soaking without Cd (Co-2), soaking in $Cd(NO_3)_2$ solution with $3x10^{-4}$ M and $5x10^{-5}$ M (Cd-1 and Cd-2) concentrations.

The decline of the amount of Put under Cd treatment in NM roots (Fig. 1) is in contrast to some previous results. Put levels increased at the concentrations of 10 and 25mM Cd in oat (*Avena sativa*) and bean (*Phaseolus vulgaris*) leaves (where the changes may be different from the roots; Weinstein et al. 1986), and at the concentration of 50 mM Cd in barley (*Hordeum vulgare*) leaves and roots (Soós 2000). However, no alteration in Put levels was detected after Cd treatment in potato leaves (Stroinski and Szczotka 1990), but the level of Spd and Spm were considerably increased. The present study was carried out on a different plant species, under unique growing conditions using root cultures, and the samples were taken at the sixth hour of the treatment, unlike to the 1-5 days incubation times used in the studies mentioned above (Weinstein et al. 1986; Stroinski and Szczotka 1990; Soós 2000).

Soós (2000) observed differences between the changes of PA levels in the root and leaves under Cd stress conditions, as it has also been revealed in the case of P deficiency (Parádi et al. 2003). Leskó and co-workers (2002) studied Cd stress in wheat (*Triticum aestivum*). According to their results, no changes in the amount of Put was observed under low Cd stress (0.1 μM Cd), but the level of Spd rose sharply in the leaves and similar to the results of the present experiment, decreased in the root (Fig. 1). Whereas when Cd was applied in high concentration (1 mM), Put level showed an increase in the root, while the amount of Spd rose only slightly (Leskó et al. 2002).

A decline was observed in the amount of the Spd showing a similar trend to the Put levels in the NM roots when treated with Cd in this study (Fig. 1). The rates of the changes in the levels of Put and Spd in the NM roots were independent from the concentration of the Cd applied and the Cd contents of the tissues (Table 1), because their amounts were the same both in the cases of Cd-1 or Cd-2 treatments (Fig. 1). Therefore, it is postulated, that like the effect of the P deficiency (Parádi et al. 2003), Cd induced changes in PA contents might be observed above a certain level of the heavy metal and they were not connected strictly to the actual external Cd concentration under the experimental conditions of this work.

According to Weinstein and co-workers (1986), the level of Spm doubled after 24 hours Cd incubation in oat and bean leaves. Further investigations may reveal whether the moderate but steady increase of Spm levels (Fig. 1) could improve under additional incubation.

The increase in the amounts of Spd and Spm probably indicates stress tolerance, while Put causes membrane depolarization and K^+ release. Put levels normally go up under many stress conditions in sensitive plants, whilst Spd and Spm accumulate in the tolerant plants (Rácz et al. 1996; Bouchereau et al. 1999). PAs participate in the quenching of free radicals, the delay of senescence and the inhibition of

Table 2. Significance (ANOVA) of effects of mycorrhizal colonization and soaking/Cd treatment on polyamine contents and ratio and their interaction in *Daucus carota* root cultures.

	Put content	Spd content	Spm content	PA ratio
Mycorrhiza	***	***	ns	***
Treatment	*	***	***	ns
Interaction	ns	***	**	ns

Levels of significance: ns-non significant, *-P≤0,05, **-P≤0,01, ***-P≤0,001.

lipid peroxidation. They retard the increase in the superoxide levels proportional to their chain length (Bouchereau et al. 1999). Based on the role PAs play and the degree of their accumulation under different stress conditions, the Put/(Spd+Spm) ratio was defined as a suitable stress indicator parameter (Minocha et al. 1997). The general difference observed between PA ratios of the NM and M roots (Fig. 1) emphasises the marked effect of the presence of mycorrhiza on the PA metabolism and indicates a lower degree of stress in the colonised roots.

To conclude, no considerable changes in the Put and Spd contents of the roots of mycorrhizal *Daucus carota* were observed after Cd treatment, they remained similar to the control values (Fig. 1). The presence of mycorrhiza seemed to have a kind of 'equalising' effect under the Cd stress against the changes in PA contents widely described in various stress conditions (Bouchereau et al. 1999). By supplying nutrients and adsorbing heavy metals, the mycorrhiza can provide a balanced environment for the roots. This 'buffer-effect' (Rivera-Becerril et al. 2002) may alleviate some of the physiological alterations induced by stress conditions. The significant interaction between the Cd treatments and colonisation supports this theory (Table 1), since the effect of Cd on the roots was reduced in the presence of mycorrhiza. Nevertheless, mycorrhiza could have also influenced the hormonal balances of roots growing on the minimal media by supplying P and other nutrients.

Finally, it is presumed, that subsequent upon the direct hormonal or the indirect nutrient supply and/or morphological effects of the mycorrhizal colonisation, the roots can get into a different state, which is reflected by the Put and Spd contents and the ratio of PAs. The observed differences in the PA contents of the *Daucus carota* root cultures may allude to an increase in the tolerance to Cd stress in M roots. Further investigation is needed to elucidate the potential ways of the improved stress tolerance of AM colonised roots.

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ARTICLE

HPLC analysis of carotenoids in four varieties of *Calendula officinalis* L. flowers

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ABSTRACT *Calendula officinalis* L. is a medicinal plant that accumulates large amounts of carotenoids in its inflorescences. The yellow-to-orange colour of inflorescences is mostly due to carotenoids and the shade is dependent on pigments content and profile.We investigated the carotenoid content and profile in four selected varieties of *Calendula*: Double Esterel Orange, Radio Extra Selected, Bonbon Abricot and Double Esterel Jaune. The total carotenoid content was evaluated spectrophotometrically and pigments were separated using chromatographic methods (CC, TLC, HPLC). An HPLC gradient system with a Nucleosil C_{18} column and a Waters PDA detector was used for separation and identification of carotenoids. The carotenoid content was higher in orange varieties: 276 mg/100 g fresh flowers for Double Esterel Orange and 111 mg/100 g fresh flowers for Radio variety. All varieties contain the same pigments but there are significant differences for the ratio between individual pigments. Orange varieties contain higher amounts of hydrocarbons: 44.5% of total carotenoid in Double Esterel Orange; while yellow varieties contain mostly oxygenated derivatives: 97% of total carotenoids in Double Esterel Jaune. The main pigments identified were: flavoxanthin, lutein, rubixanthin, β-carotene, γ-carotene and lycopene. The cultivation of orange varieties is recommended especially when the pharmacological products for skin protection are envisaged.
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KEY WORDS

Calendula officinalis L
carotenoids
HPLC
chromatography

Calendula officinalis L. is one of the most known and used medicinal plants, that is widely cultivated both for obtaining extracts used in phytotherapy and ornamental purposes. The chemical composition of the species and of its varieties is very complex. Among the biologically active compounds we can mention the isoprenoids and derivatives, the phenolic compounds, the polysaccharides, the nitrogen compounds, etc. The extracts obtained from *Calendula* possess a wide range of pharmacological effects: wound-healing, anti-inflammatory, antibacterial, immunostimulative, antitumoral, etc.. The shade depends on the carotenoids' composition; the yellow varieties are rich in xantophylls (oxygenated derivatives), while orange varieties contain higher amounts of hydrocarbons (Toth and Szabolcs 1981; Milborrow et al. 1982; Petri and Lemberkovics 1994). Some works refer to qualitative aspects (separation and identification of carotenoids) and others to quantitative determination (total carotenoid content; Bomme et al. 1997; Piccaglia et al. 1997). Recently, the carotenoids composition of petals, pollens, leaves and steams of *Calendula* was investigated by HPLC (Bako et al. 2002). However, there are no systematic studies yet using a genetically controlled material regarding both aspects.

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Carotenoids are known as biologically active compounds with multiple applications in therapy. Beside the provitamin A activity (of some pigments), it was proved that carotenoids have a favorable effect on the epitelisation process, influencing the cell cycle progression of the fibroblasts (Stivala et al. 1996). Carotenoids act as photoprotective agents (depending on the dose) and may reduce the risk of sunburns, photoallergy and even some types of skin cancer (Fuchs 1998; Lee et al. 2000). Lycopene is an active inhibitor of tumour cells proliferation (Levy et al. 1995), but oxygenated carotenoids can also have biological properties due to their antioxidant properties (Woodall et al. 1997; Smith 1998).

The aims of our study were to determine the carotenoid content in four selected varieties of *Calendula officinalis* L, the separation and identification of carotenoids and the determination of the carotenoids profile in these varieties by HPLC-PDA.

Materials and Methods

Biological material

The carotenoids analyses were made on four selected varieties, differentiated by colour, bought in specialized stores in France: "Esterel Double Orange" – with big, dark orange inflorescences, "Esterel Double Jaune" – with big, lemon yellow inflorescences, "Radio Extra Selected" - tall, with

Table 1. The total carotenoid content in some *Calendula officinalis L.* varieties.

Variety	Colour	Carotenoid amount (mg/100g fresh flowers)
Bonbon Abricot	Yellow-orange	48.2
Double Esterel Jaune	Lemon yellow	97.0
Radio Extra Selected	Orange	111.8
Double Esterel Orange	Dark orange	276.0

orange inflorescences, "Bonbon Abricot" – small, with yellow-orange inflorescences. The plants were cultivated in experimental fields of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca.

Total carotenoids were extracted from 10 g fresh inflorescences with a mixture of methanol/ethyl acetate/petroleum ether (1:1:1, v/v/v) containing BHT as antioxidant and calcium carbonate. The total carotenoid content was estimated spectrophotometrically at 450 nm using a Perkin-Elmer Spectrophotometer.

The extract was dissolved in diethyl ether and saponified with 30% methanolic KOH at room temperature in dark. For the removal of soaps and alkalies, the solution was washed many times with a sodium chloride-saturated solution and distilled water. The organic layer containing carotenoids was dried over anhydrous sodium sulphate and evaporated to dryness. The samples were kept under nitrogen, at – 20° C until further utilization and were filtered through 0.45 mm Whatman filters prior HPLC analysis (Britton et al. 1995).

A part of oleoresin dissolved in light petroleum was subjected to column chromatography on aluminium oxide grade III (100x10 mm). For removal of neutral lipids, the column was washed twice with light petroleum. Three fractions were collected separately 1) with petroleum ether, 2) with 50% diethylether in petroleum ether and 3) with 100% diethyl ether to 20% ethanol in diethyl ether. The fraction collected from alumina column was further subjected to TLC on silica thin layer plates using in parallel standards of β-carotene, β-cryptoxanthin, and lutein. The bands separated on silica plates were scratched out and separated on magnesium oxide-kieselguhr plates, in order to obtain pure compounds.

HPLC-DAD of all samples was performed on a system with Waters 990 PDA detector, Kontron 322 pumps and controller, a Rheodyne 7152 injection valve with a 20 ml loop and a reversed phase C 18 column Nucleosil ODS (250 x 4,6 mm), 5 μm. The mobile phase consisted of mixtures of acetonitrile: water (9:1, v/v) with 0.25% triethylamine (A) and ethyl acetate with 0.25% triethylamine (B). The gradient started with 90% A at 0 min to 50% A at 10 min. The percentage of A decreased from 50% at 10 min to 10% A at 20 min. The flow rate was 1 ml/min and the chromatogram was monitored at 450 nm.

Identification of carotenoids was based on co-chromatography using standards, chemical tests on pure compounds and characteristics of UV-VIS spectra recorded by PDA detector and Perkin-Elmer spectrophotometer for pure compounds.

Results and Discussion

Quantitative determination of carotenoids

The results of quantitative determination of total carotenoid contents in the four investigated varieties are presented in Table 1.

The richest variety in carotenoids content from flowers was Double Esterel Orange, with a total content of 276 mg/

Table 2. Carotenoid composition in inflorescences of Calendula officinalis L. varieties.

Pigment	Nr. of Pigment on HPLC chromatogram	Double Esterel Orange %	Radio Extra Selected %	Bonbon Abricot %	Double Esterel Jaune %
Neoxanthin	1	0.92	1.71	2.84	1.74
Luteoxanthin + Auro	8	8.9	11.3	15.43	18.97
Antheraxanthin	9	2.09	4.31	4.56	6.83
Flavoxanthin	10	14.1	17.4	35.42	42.05
Mutatoxanthin	11	0.38	-	2.17	-
Lactucaxanthin	12	4.49	8.02	-	11.31
Lutein	3	9.18	11.38	8.27	12.29
Zeaxanthin	4	0.11	0.23	-	0.15
Rubixanthin	13,14	14.36	7.27	4.58	-
Lycopene	15	14.03	5	0.57	-
γ-carotene	16	12.15	6.15	5.11	-
α-carotene	17	0.98	1.15	1.89	0.2
β-carotene	7	16.68	17.51	10.31	2.37

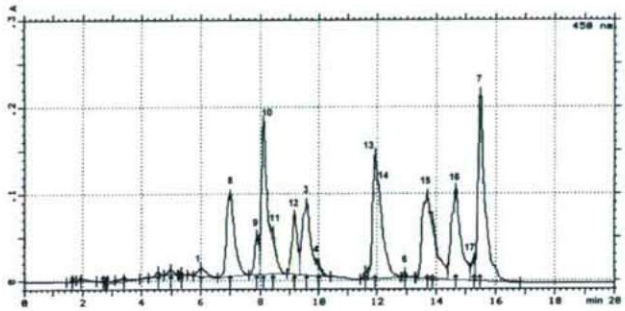

Figure 1. The HPLC chromatogram of total saponified extract from Double Esterel Orange variety.

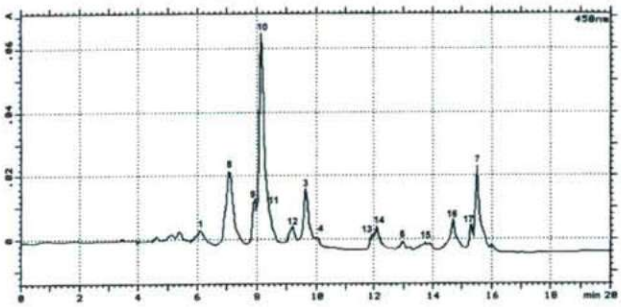

Figure 3. The HPLC chromatogram of total saponified extract of Bonbon Abricot variety.

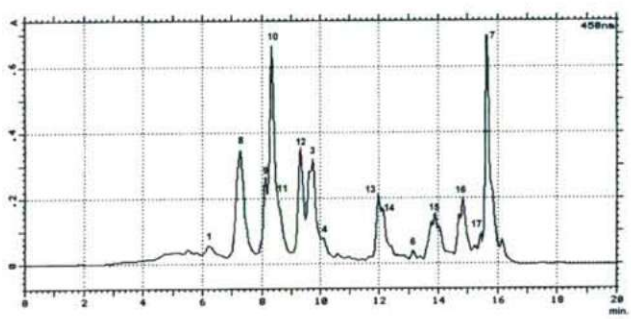

Figure 2. The HPLC chromatogram of total saponified extract of Radio Extra Selected variety.

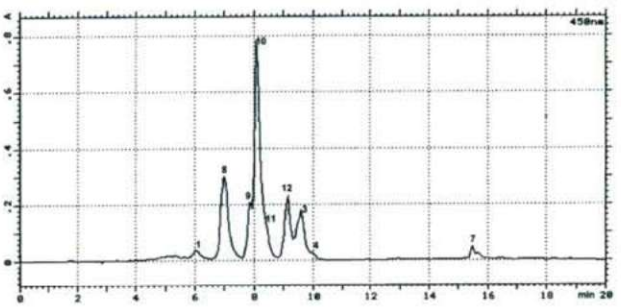

Figure 4. The HPLC chromatogram of total saponified extract of Double Esterel Jaune variety.

100 g fresh flowers. The carotenoid content in petals and tubular flowers of Radio Extra Selected was also determined. The highest amount of carotenoids was found in petals (103,8 mg/100 g), while tubular flowers contains only 8 mg/100 g fresh material. The values of quantitative determinations are very different from one variety to another. The amount of carotenoids increases with colour intensity, the dark orange variety being the richest one. The values we obtained are comparable to those indicated in literature (Goodwin 1980; Neamtu et al. 1981).

The separation and analysis of carotenoid pigments

The total saponified extracts obtained from the four varieties were separated using chromatographic methods (LC – Al_2O_3, TLC - silica and magnesium oxide, HPLC-PDA) in order to identify the carotenoid pigments. The carotenoids separated and identified by HPLC and the percentages resulted from peak integration are presented in Table 2.

We observed that all the varieties contain the same pigments, but there are significant differences for the ratios between individual pigments.

The first observation is the existence of a direct relationship between the colour of the inflorescences and the nature of carotenoids. The variety Double Esterel Orange (dark orange) is the richest in hydrocarbons and rubixanthin. We also observed a high content of lycopene (14%), and γ-carotene (12%). In Radio variety (orange), these two hydrocarbons have significant lower values: 5% for lycopene and 6 % for γ-carotene. Bonbon Abricot variety contains important amounts of β-carotene, but very low amounts of γ-carotene and it is especially low in lycopene (0.57% from total carotenoids).

The lemon yellow variety, Double Esterel Jaune, does not contain lycopene and γ-carotene while α-carotene and β-carotene together represent 3% of total carotenoid contents.

The rubixanthin (3-hydroxy-β, ψ-carotene, a derivative of γ-carotene) content varies in the same direction as hydrocarbon contents. The percentages of γ-carotene and rubixanthin are directly proportional, but we cannot observe the same relationship between β-carotene and their monohydroxy derivative, β criptoxanthin. The intensity of the orange colour of Calendula is determined by the amount of lycopene, γ-carotene, β-carotene and rubixanthin; these pigments are responsible for the orange or even red colour of vegetal tissues.

We have found a wide range of oxygenated carotenoids in all varieties investigated. The most important ones, by

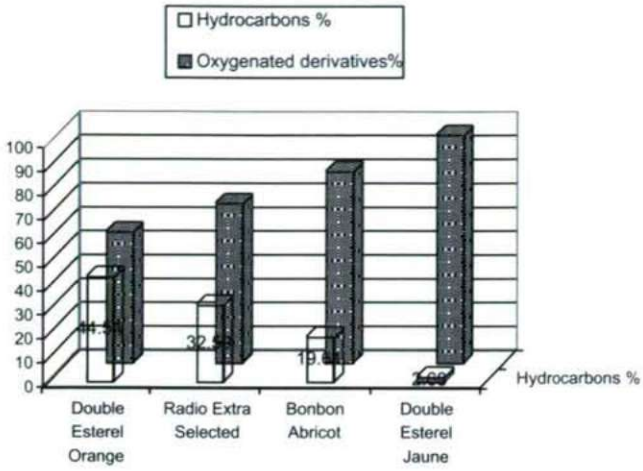

Figure 5. Hydrocarbons and oxygenated compounds content in different varieties of Calendula.

quantitative point of view, are the flavoxanthin and the lutein. Except for the rubixanthin, all varieties contain the same xanthopylls. The great majority of xanthophylls have a β-ε structure: flavoxanthin, lutein, luteoxanthin. Zeaxanthin (3,3'- dihydroxy-β, β- carotene) is present in small amount, as well as their epoxides: anteraxanthin, mutatoxanthin and auroxanthin.

The increasing percentage of the oxygenated compounds is accompanied by a decrease in hydrocarbons (Fig. 5). From a quantitative point of view, orange varieties are the richest in carotenoids and they contain both hydrocarbons and oxygenated derivatives.

Conclusion

All varieties of *Calendula* are rich in carotenoids. Double Esterel Orange and Radio varieties contain the most important amounts of pigments. The content and the distribution of carotenoids seem to be strongly influenced by the nature of biological material. The orange varieties contain important amounts of hydrocarbons, with provitamin A activity, while yellow varieties contain mainly oxygenated compounds. In the orange varieties, a preferential biosynthesis of hydrocarbons with ψ-ψ and β-ψ structure and of monoxantophylls with β-ψ (or α-ψ) structure was noted.

The Double Esterel Orange variety has a special profile of carotenoids and contains a great amount of pigments. Their composition recommends this variety for using in pharmacological products designated to the skin protection.

Acknowledgments

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ARTICLE

Clinical and genetical aspects of autosomal dominantly inherited osteogenesis imperfecta tarda

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ABSTRACT Osteogenesis imperfecta (OI) tarda dominant type is caused by mutations in the type I collagen genes, COL1A1 and COL1A2. The essence of our haplotype analysis of osteogenesis imperfecta (OI) was to get information about the value of 8 short tandem repeat (STR) markers for the segregation of COL1A1 and COL1A2 genes on the 12 OI pedigrees and to delimit the place of mutation to one locus. The molecular genetic analysis supported the linkage to COL1A1 gene in 6 families, and in 4 families with type I B, and in one family with type III B the linkage to COL1A2 gene was supported. One patient had type IV A, where linkage to COL1A1 gene had been excluded, and haplotype analysis for COL1A2 was non conclusive in six families. As both genes consist of more than 50 exons, the haplotype analysis is very important before direct mutation screening. To achieve the maximum theoretical LOD scores for the haplotype analysis, more STR markers are needed as in many cases our markers were non informative.

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KEY WORDS

Osteogenesis imperfecta tarda
molecular genetics
short tandem repeat

Osteogenesis imperfecta (OI) is characterised by brittle bones, pathologial fractures, blue sclera, dentinogenesis imperfecta (with or without) and caused by mutations in the type I collagen genes, COL1A1 and COL1A2.

All types of osteogenesis imperfecta are caused by structural or quantitative defects in type I collagen, the primary component of the extracellular matrix of bone and skin. In about 10% the of clinically indistinguishable cases, no biochemical defect of collagen protein can be demonstrated. It is not clear whether these cases represent limitations in biochemical detection or genetic heterogeneity of the disorder.

OI is an autosomal dominant disorder that occurs in all racial and ethnic groups. The incidence of detectable OI in infancy is about 1 in 20,000. There is a similar incidence of the mild form, type I OI.

Pathology: The collagen structural mutations cause OI bone to be globally abnormal. The bone matrix contains abnormal type I collagen and relatively increased levels of types III and V collagen. In addition, several non collagenous proteins of bone matrix are found in reduced amounts. The hydroxyapatite crystals deposited on this matrix are poorly aligned with the long axis of fibres.

Pathogenesis: Type I collagen is a heterotrimer, composed of two α-1 (I)-chains and one α2(I)–chain. The chains are synthesized as pro collagen molecules with short globular extensions on both ends of the central helical domain. The helical domain is composed of uninterrupted repeats of the sequence Gly-X-Y, where Gly is glycine, X is mostly proline, and Y is mostly hydroxyproline. The presence of glycine at every third residue is cruical to helix formation because its small side chain can be accomodated in the spatial constraints of the interior of the helical trimer. Concomitant with helix assembly and formation, the chains are glycosylated at lysine residues.

The collagen structural defects are of two types: 85% are point mutations causing substitutions of glycine residues by other amino acids, 12% are single exon splicing defects.The clinically mild type I OI has a quantitative defect with mutations that cause one a1(I) allele to be functionally void. These patients make a reduced amount of normal collagen. The relationship between genotype and phenotype remains elusive for the structural mutations. Lethal and nonlethal mutations occur with about equal frequency on both chains. For α2(I) mutations, lethal and nonlethal mutations occur in alternating regions along the chain. For mutations on the α1(I)-chain, no model adequately predicts the phenotype. A minority of OI cases with apparent recessive inheritance are due to parental mosaicism and are also dominant.

Pace et al. (2002), identified a mutation in the carboxyl-terminal propeptide coding region of one COL1A1 allele in an infant who died with an OI phenotype that differed from the usual lethal form and had regions of increased bone density.

Linkage studies were performed for genetic markers from candidate intervals known to contain genes responsible for

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dentinogenesis imperfecta (DGI) on chromosomes 4q, 7q, and 17q. Conclusive evidence for linkage of DGI was obtained for genetic markers on chromosome 17q21-q22 (DLX-3, Z(max) = 5.34, theta = 0.00; Pallos et al. 2001). This was the first report of joint pain associated with a COL1A1 mutation and DGI. The mild skeletal features and reduced penetrance of the non-dental findings illustrate the importance of genetic evaluations for families with a history of DGI.

McKenna et al. (2002) determined whether milder abnormalities in COL1A1 expression might account for the development of otosclerosis in the 7 clinical cases that did not reveal evidence of zero expression by the gel technique. Of the same 2 cases of otosclerosis that demonstrated evidence of zero expression by gel electrophoresis, both were found to have significant differences in COL1A1 mRNA expression by the Taqman analysis. The remaining 7 cases revealed equal expression of the two COL1A1 alleles similar to that seen in controls. These results suggest, that mutations in COL1A1 which are similar to those in type I osteogenesis imperfecta may account for a small percentage of cases of otosclerosis, and that the majority of cases of clinical otosclerosis are related to other genetic abnormalities that have yet to be identified.

In summary, there are two known loci (COL1A: at chromosome 17, 17q21.31-q22; COL1A2: at chromosome 7, 7q22.1) of osteogenesis imperfecta type I; 70-80% of these patients carry mutations in one or both of these genes.

The aim of our study was to analyse the segregation of the autosomal dominant osteogenesis imperfecta type I in some Hungarian families and to evaluate the usefulness of short tandem repeat markers (STR) for the linkage analyses of certain loci of collagen gene.

Materials and Methods

In our retrospective study we investigated 18 families with autosomal dominant inherited osteogenesis imperfecta tarda (OIT) type I, III and IV from the Pediatric Department Szeged and 12 families from the Semmelweis University Department of Orthopedics, Budapest. Diagnostic criteria for OIT were pathological bone fractures, blue sclera, dentinogenesis imperfecta, scoliosis (w/wo). Type II (lethal) did not occur.

We chose 4-4 short tandem repeat (STR) markers (Table 1) for the haplotype analysis of the two loci (COL1A1 and COL1A2). The markers were D17S788, D17S790, D17S943, D17S1795 for COL1A1, and D7S657, D7S527, D7S2482, D7S1820 for COL1A2. The touch down PCR amplifications were carried out by the following program: initial denaturation at 94°C for 5 min followed by 10 cycles denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec (-0.5°C per cycle) and extension at 72°C for 30 sec continued for 25 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 30 sec followed by a final extension at 72°C for 10 min for all markers except for D17S943 where we had the same program but the annealing temperatures were 5°C higher, respectively. The PCR products were run on 8% polyacrylamide (PA) gel and visualized by ethidium bromide with an AlphaImager, AlphaEase 5.5 gel documentation system (Fig. 1).

Results

Twelve OIT families, 3-7 family members per family, including 2-4 members affected by autosomal dominant inheritance (AD) per family, had been genotyped by STR markers for the COL1A1 and COL1A2 loci. The clinical and genetical data of our OIT patients have been summarized in Table 2. Eight out of twelve OIT families had type I, among them 5 families had type I B, the molecular genetic analysis supported the linkage to COL1A1 gene in 6 families, and in 4 families with type I B and in one family with type III. B the linkage to COL1A2 gene was supported.

One patient had type IV A, where linkage to COL1A1 gene had been excluded, and haplotype analysis for COL1A2 was non conclusive. The K. brother's had type IV B with COL1A1 mutation.

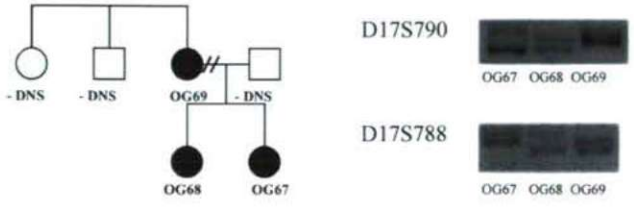

Figure 1. Haplotype analysis of the family of M. Sz. The amplified PCR products were run on 8% PA gel. We could exclude the COL1A1 locus with several markers as affected children inherited different alleles from the affected mother. The STR markers of COL1A2 locus (data not shown) did not disagree with the segregation to COL1A2.

Table 1. STR markers for the haplotype analysis of COL1A1 and COL1A2 loci

D17S788F	5' CTA GGC AGC CAC TAC CAA AT
D17S788R	5' CAG CAT CTT TGC TAT AAG CAT C
D17S790F	5' AGG AAA AAT GAG TGG ACC AT
D17S790R	5' AGC TGG GTT ATT GTT TTT CC
D17S943F	5' GGT GGA GGG AGT AGA AAG AA
D17S943R	5' TAT GGC TGT CTC ATT CCA AC
D17S1795F	5' AGT GCC AGA GATATA CCG TG
D17S1795R	5' GTC TGC AAG GCA AGT TGT C
D7S657F	5' TCA ATC ACC ACT TAC ATG CA
D7S657R	5' AGA GAA GTG GTG TCA CTT GG
D7S527F	5' TGT TTC TTC AAG GTA GTC
D7S527R	5' TAA CAG AGG CAT GAA AAC CA
D7S2482F	5' TTA ATC CCA CAG GAA TGA ATG
D7S2482R	5' TGG CTC TAG AAT TCT GAA TGG
D7S1820F	5' TGA ATG ACT TTG GTG AGT ATG C
D7S1820R	5' ACC TCA AGC AGA ACA CTT GC

Table 2. Clinical and genetic data of patients with osteogenesis imperfecta tarda.

Name	Gen-der	Age (y)	Fracture	Onset of fr. (y)	Blue sclera	Dentino-genesis imp.	Scoliosis	Affected family members	Sillence type	Molecular genetic data supported	excluded
Ms. N. H.	F	19	8X	1.5	4+	neg.	pos.	sister, father, paternal grandmother	IV.		
Mrs. G. Sz.	F	20	8-10X		3+	neg.	neg.	mother, sister	IV.A		COL1A1, COL1A2 non informative
Ms. S.	F	22	5X	8	2+	pos.	neg.	mother, brother	I. B		
R.S.	M	25	2X	10	1+	pos.	neg.	mother, sister	I. B		
Ms. A. Cs.	F	13	4-5X	6	2+	pos.	neg.		I. B	COL1A2	COL1A1 non informative
Ms. A.P.	F	3	2X	1.5	1+	pos.	neg.	mother incompl.	I. B	COL1A2	COL1A1 non informative
Ms. D.J.	F	6	3X	3y	1+	pos.	neg.	father (4-5 fra.), grandmother 14 fra.	I. B	COL1A1	COL1A2 non informative
Mr. R.K.	M	28	12X	4y	2+	pos.	neg.	brother, father	IV.B	COL1A1	COL1A2 non informative
Mr. N. K.	M	32	20X	10m	2+	pos.	neg.	father, paternal uncle	IV.B	COL1A1	COL1A2 non informative
Mr. Zs. F.	M	26	4X	2,5y	1+	pos.	neg.	father (cox arthrosis), paternal grandmother	I. B	COL1A2 (2A)	COL1A1 (1A)
Mr. A.F.	M	56	5X	14y	2+	pos.	neg.	mother	I. B	COL1A2 (2A)	COL1A1 (1A)
N.R.	M	8	2-3X	15m	1+	pos.	neg.	mother incompl.oi.	I. B		
K. T.	M	12	2-3X	i.ut.	1+	pos.	pos. operated	new mutation ?	III.		
Ms. K. É.	F	22	>10X	i.ut.	4+	pos.	pos. operated	intrauterin fractures	III. B	COL1A2	COL1A1
Mrs. B.	F	44	8-10X		1+	pos.	neg.	father, brother	I.	COL1A1	COL1A2 non informative
Mr. L.O.	M	39						sister, father, paternal grandmother	I.	COL1A1	COL1A2.
I. K.	M	7						sister, mother inc.OG..	I.	COL1A1	COL1A2 non informative
Ms. M. K.	F	14	5-6X	3y	2+	pos.	neg.				

A = absence of dentinogenesis imperfecta
B = presence of dentinogenesis imperfecta
* = the fetus and the Mr. A. P. inherited the same COL1A2 allele, probably he has been an affected person for OI. No interruption.

One female patient had type III B with intrauterine fractures and very severe short state (115 cm) and clinical prognosis with multiplex extra uterine fractures. She had been operated several times, and developed kyphoscoliosis. She completed her secondary school with excellent marks, and she is unable to walk. The affection of COL1A2 has been supported and COL1A1 gene has been excluded.

Thus the molecular genetic data linkage to COL1A1 gene was confirmed in 6 families, and in 3 families this gene was excluded. The linkage to COL1A2 was confirmed in 3 families-with type I B (n=2) and type III (n=1)-and in one family the locus was excluded.

The STR markers for COL1A2 gene were non informative in 6 families.

Discussion

Because of the low number of patients in several of our families, we were able to carry out the haplotype analysis in 12 families only.

Tsipouras et al. (1984) performed linkage analysis of the four RFLPs of the COL1A2 in families with mild forms of osteogenesis imperfecta and in a family with Marfans syndrome. In 2 of the OI positive families lod scores were found. In three other families the osteogenesis imperfecta phenotype segregated independently of the polymorphisms. Accumulative lod score from these 3 families was -4.3 at recombination fraction 0.05, and close linkage is excluded. The data from these 5 families with OI support the hypothesis that the mild dominant osteogenesis imperfecta is heterogeneous at the gene level. Skyes et al. (1986) found a polymorphism in the COL1A1 gene, and they have used this together with the polymorphisms at the COL1A2 gene in linkage studies in 11 families with dominant OI. In each family the OI gene was inherited with one or the other collagen locus. OI phenotype IV seemed to segregate with the COL1A2 gene, which is in agreement with the COL1A2 gene in four families and with COL1A2 in one family (Borresen 1986).

Comparison of phenotypic features with the concordant collagen locus showed that in four pedigrees with OI Sillence type I segregated with COL1A1, while two pedigrees with OI Sillence type I and OI type IV segregated with COL1A2.

In the five of our OI type I families the linkage to COL1A1 gene have been supported by the haplotype analysis. COL1A1 locus dimorphisms A/MspI, B/RsaI and F/MnlI, showed PIC values of 0.327, 0.191 and 0.366, respectively, giving a combined PIC of 0.656 at the locus, while COL1A2 locus dimorphisms C/EcoRI, D/MspI and E/RsaI RFLPs had PIC values of 0.357, 0.168 and 0.331, respectively, giving a combined PIC of 0.655 at the locus (Benuslene and Kucinskas 2000).

The cell surface expression and functional properties of TGF-β receptors I, II and III on osteoblasts were compared to healthy controls. The human osteoblastic cells from investigated patients with OI all have an elevated number of cell surface receptors for TGF-β, without any evidence for a transcriptional regulation of TGF-β receptor II. On the functional level, there is some evidence for an impaired adaptive behavior of receptor presentation, whereas receptor affinity is unchanged (Gebken et al. 2000).

Apart from the affected bone density, the other main manifestation of OI is the dentinogenesis imperfecta and the otosclerosis.

Recurrent mutations in the COL1A2 gene (1121 G>T, Gly 238 cys) in patients with osteogenesis imperfecta type III were reported by Trummer et al. (2001). A new recurrent point mutation in the COL1A2 gene was found in a patient with type III OI. A G-to-T transversion in nucleotide position 1121 leads to an amino acid substitution Gly238Cys. This was the first report on the most N-terminal cysteine substitution in COL1A2 reported to date. Until now, at this position, only serine substitutions were observed five times in unrelated patients showing a highly variable expression of OI. Our patient (Ms. K. É.) with type III is a candidate for COL1A2 gene mutation because her STR data were supportive for COL1A2 gene affection.

Prenatal diagnosis of a novel COL1A1 mutation in osteogenesis imperfecta type I carried through full term pregnancy was published by Ries et al. (2000). The father has OI type I, with a novel mutation in the COL1A1 gene: a C to T change at position c3076 (c.3076C—>T) leading to a change of arginine at codon 848 to a stop codon (R848X). Prenatal diagnosis by chorionic villous sampling (CVS) was performed during the fourth pregnancy, and revealed that the fetus is a carrier of the same COL1A1 mutation. The parents elected to carry the pregnancy to term, and a male child with mild OI was born.

The only serious attempts to show linkage in OI have shown that the disease is linked to type 1 collagen genes in all studied families in which it segregrates as a clear mendelian dominant trait. For prenatal diagnosis the probability that a new family is linked can be taken as greater than 0.95. Some phenotype correlations, notably between the OI type IV phenotype and linkage to COL1A2 and between presenile hearing loss in OI type I and linkage to COL1A1, can be used to improve risk estimates substantially in families where there are no segregation data to distinguish whether COL1A1 or COL1A2 is the mutant locus (Sykes 1993).

The segregation of COL1A1 and COL1A2, the two genes which encode the chains of type I collagen, was analyzed in 38 dominant OI pedigrees by using polymorphic markers within or close to the genes. This was done in order to estimate the consistency of linkage of OI genes to these two loci. None of the 38 pedigrees showed evidence of recombination between the OI gene and both collagen loci, suggesting that the frequency of unlinked loci in the population must be low. From these results, approximate 95%

confidence limits for the proportion of families linked to the type I collagen genes can be set between .91 and 1.00. This is high enough to base prenatal diagnosis of dominantly inherited OI on linkage to these genes even in families which are too small for the linkage to be independently confirmed to high levels of significance. When phenotypic features were compared with the concordant collagen locus, all eight pedigrees with Sillence OI type IV segregated with COL1A2. On the other hand, Sillence OI type I segregated with both COL1A1 (17 pedigrees) and COL1A2 (7 pedigrees). The concordant locus was uncertain in the remaining six OI type I pedigrees. Of several other features, the presence or absence of presenile hearing loss was the best predictor of the mutant locus in OI type I families, with 13 of the 17 COL1A1 segregants and none of the 7 COL1A2 segregants showing this feature (Sykes et al. 1990).

The essence of our haplotype analysis of OI was to get information about the value of 8 STR markers for the segregation on the pedigrees and to delimit the place of mutation to one locus. As both genes consist of more than 50 exons the haplotype analysis is very important before direct mutation screening. From our study we can conclude that the analysis of AD OI families is problematic as in most cases the number of affected people and informative members in the families are few. To achieve the maximum theoretical LOD scores for the haplotype analysis more STR markers are needed as in many cases our markers were non informative. However by the data of HGP there are limited numbers of available STR markers with high allele frequencies in 3-4 cM proximity of COL1A1 and COL1A2 loci.

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ARTICLE

Consanguinity, genetic disorders and malformations in the Iranian population

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ABSTRACT The present study focuses on the effect of parental consanguinity on genetic disorders in the Iranian population, which is predominantly Muslim and where consanguineous marriages are quite common. Data were collected from three genetic centers from different areas of Tehran. Out of 800 affected subjects nearly 44% were born to consanguineous parents. While 37.8% of them were born out of parallel- cousin marriages, 28.9% were from cross-cousin alliances. Frequency of occurrence of genetic disorders was twice in children born to parallel-cousin parents as compared with those occurring out of cross-cousin marriages. Psychomotor retardation (14.3%), primary amenorrhoea (11.2%), and mental retardation (6.6%) topped the list of disorders encountered in children born to consanguineous parents. Cases of phenylketonuria were encountered exclusively in children of consanguineous couples. Of the patients having positive family history of genetic disorders, 93% had consanguineous parents. Two points emerge from the present study: that related parents, whatsoever the relationship, are more likely to have children with genetic defects; consanguineous couples who already have an affected child are 13 times more likely to have another affected child.
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KEY WORDS

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Bob Edwards, the world-renowned embryologist, while speaking at the annual meeting of the European Society of Human Reproduction and Embryology in France in July 1999 said: "Giving birth to a disabled child is a sin no parent should commit in the present era when prenatal screening for genetic diseases is increasingly available. It is the moral responsibility of the parents too not to have a child that carries the heavy burden of genetic disease. We are entering a world where we have to consider the quality of our children..."

Going by the old adage that prevention is always better than cure, present study explores once again the role of parental consanguinity often associated with a higher frequency of genetic disorders and congenital malformations in their progeny (Verma and Mathew 1983; Mukherjee 1994; Afrooz 1996). It is well supported by similar studies carried out focussing on individual malformations reported in the couples closely related (Kesavan et al. 1978; Kabiri 1995; Karimi-Nejad 1995).

In the Iranian society, which is, predominantly a muslim society where consanguineous marriages are quite common, few such studies focussing on consanguinity and genetic disorders in Iranian population are available. Jorjani (1994) selected a religious isolate group known as the Hamedanis (originally from Hamedan Province in the western part of Iran where consanguineous marriages are a norm; they migrated to India and settled down at Junnar district of Maharashtra about 400-500 years ago). It is assumed that intense level of consanguinity and inbreeding in this population must have lead to an increase in homozygosity, resulting in an increase in genetic anomalies among the Hamadanis. Another study conducted in a single village in the suburb of Hamedan reported that 60 mentally retarded children were born in the year 1995 alone there (Shariati 1996). In some other villages near Hamedan, altogether 1050 mentally retarded, deaf, blind and epileptic babies were born in the same year. Alarmed by this statistics, immediately a genetic center was opened which became functional the same year as the need was felt very strongly.

Karimi-Nejad (1995), upon comparing the frequencies of congenital malformations and multifactorial diseases in two thousands couples, studied the consanguineous couples whose inbreeding coefficient were equal or greater than 1.64. He concluded that considering the studies conducted in Shiraz and Turkey, a total of one-fourth of marriages in Iran are consanguineous and as a result congenital malformation and genetic disorders which are inherited, were higher in Iran because of high degree of consanguinity. Moreover, the frequency of malformations and diseases caused by inheritance in consanguineous marriages was twice in frequency than in non-consanguineous marriages. He commented that in cases where inherited factors were more influential, this difference was more obvious, but it was lesser in the type of

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Table 1. Consanguinity of parents of the patients

Consanguinity	Number	Frequency
parallel cousin	132	37.8
cross cousin	101	28.9
distant relative	116	33.2
sub total	349	43.6
non-consanguineous	451	56.4

diseases where the environmental factors were more effective to the causation of the disease, and vice versa. In the same year, Kabiri (1995), in his article on the frequency of phenylketonuria (PKU) in consanguineous couples in Iran, reported that the disorder was three times more frequent in children born out of consanguineous marriages than among the non-consanguineous couples. Earlier Ameli and associates (1988) reported that out of 200 cases of PKU studied by them in seven years' span (1979-1986) in a local hospital in Tehran, the frequency recorded was 1 in 4000 live births as against 1 in 10,000 live births in the US. Iran had the highest figures when compared to some other countries like North Ireland (1 in 4,500), Greece, Poland and Scotland (1 in 8,000) and Japan and Denmark (1 in 60,000), the last two countries having implemented the most advanced preventive measures to combat PKU (Ghiyasvand 1995).

In Iran the present state for diagnosis and prevention of PKU is like it was in developed countries in 1950s when they did not have advanced screening tests. The average age for diagnosis of PKU in Iran is two years, and unfortunately, by then the child is already mentally retarded and beyond cure. In another study on consanguineous marriages in different states of Iran more than 54,000 families were screened and a high rate of infant mortality was reported (Meshkani and Meshkani 1997). Afrooz (1996) based on his study concluded that " ...the rate of malformations in consanguineous marriages (in Iran) is five times more than in the non-consanguineous marriages. Out of every 100 non-consanguineous marriages two babies are born with a defect but in consanguineous marriages this rate increases to 10 defective babies."

Most of these studies in Iran have been conducted in the last decade or so and are limited to hospital birth records. The majority deals with a single gene disorder. Even though a few genetic centers have become operational in the recent past, not many systematic and well planned study has been published so far.

In the view of the limited work in this high-risk population, present research was taken up in three genetic centres in Iran focussing on the prevalence of congenital malformations and genetic diseases in the consanguineous marriages in Iranian population. An attempt was also made to examine the relationship of pattern of consanguinity of the parents of the patients, and the frequency of genetic anomalies in the offsprings.

Materials and Methods

Tehran the capital of Iran has better medical facilities as compared to other cities and rural areas of Iran. A few government hospitals have genetic clinics and diagnostic facilities for genetic diseases. There are also a few private genetic centers where karyotyping is done, which are more expensive, charging about two times of the regular fee at the government centers. Many affected individuals are brought to Tehran from neighboring towns for diagnosis, and with a hope for treatment. Data for the present study were collected from three genetic centers in Tehran. These centers, Karimi-Nejad Pathological and Genetic Center, Shahid Akbarabaadi Hospital, and Pediatric Medical Center were located in north-west, south and south-west of Tehran respectively.

Detailed information on 850 subjects was collected using an interview schedule and through daily interviews with the

Table 2. Type of consanguinity and some major disorders in the offspring

	Consanguineous parents									
Disorder	Parallel cousin		Cross cousin		Distant relative		Total consanguineous		Non-consanguineous	
	No	%	No	%	No	%	No	%	No	%
psycho. ret.	23	17.4	14	13.9	13	11.2	50	14.3	22	4.9
down synd.	15	11.4	7	6.9	20	17.2	42	12	134	29.7
prim. amen.	13	9.8	10	9.9	16	13.8	39	11.2	48	10.6
mental ret.	11	8.3	6	5.9	6	5.2	23	6.6	18	4
growth ret.	5	3.8	5	5	6	5.2	16	4.6	7	1.5
turner synd.	2	1.5	6	5.9	8	6.9	16	4.6	25	5.5
sec. amen.	6	4.5	5	5	3	2.6	14	4	14	3.1
fragile-X	5	3.8	2	2	6	5.2	13	3.7	10	2.2
microcephaly			3	3	4	3.4	7	2	3	0.7
multiple. cong. anomalies	2	1.5	1	1	2	1.7	5	1.4	3	0.7
klinefelter	2	1.5	1	1	2	1.7	5	1.4	3	0.7
P. K. U.	1	0.8	1	1	2	1.7	4	1.1		
others	85	64.4	40	39.6	28	24.1	115	33	164	36.4
total	132	16.5	101	12.6	116	14.5	349	43.6	451	56.4

Table 3. Previously affected siblings of the patients

Number of affected	Relationship of parents: Parallel cousin No.	%	Cross cousin No.	%	Distant relative No.	%	Total (CM) consanguineous No.	%	Non-(NCM) consanguineous No.	%	Total CM+NCM No.	%
1	13	37.4	7	20	15	42.85	35	87.5	3	6.98	38	88.37
2	2	50	1	25	1	25	4	10			4	9.3
4	1	100					1	2.5			1	2.33
Total	16	37	8	18.6	16	37	40	93	3	7	43	100

patients and accompanying persons. Their medical files and reports of their karyotyping tests were studied. Discussions with the consulting doctors were very informative. Fifty cases had to be excluded for various reasons and after the karyotyping results became known.

Results and Discussion

The majority of the patients coming to the genetic centers were from urban areas (99%) and a few were from neighboring villages. A high level of consanguinity (43.6%) was reported among the parents of the patients. While 38% of these affected children were born to parallel cousin parents, 33.2% were from marriages of distant relatives, and 29% of them were born out of cross-cousin alliances (Table 1).

Psychomotor retardation (14.3%), primary amenorrhoea (11.2%) and mental retardation (6.6%) topped the list of disorders encountered in children born to consanguineous parents. All the phenylketonuria patients were born, exclusively, to consanguineous parents (Table 2). Nearly 21% of the mentally retarded (including cases of psychomotor retardation) were born from consanguineous alliances as compared to only 8% from non-consanguineous parents. An earlier study on parental consanguinity and mental retardation reported a higher frequency of mental retardation in the consanguineous parents (Narayan and Rama Rao 1978).

It was not surprising that more than double (29.7%) the frequency of Down syndrome children were born to non-consanguineous patients as compared to only 11% of them born to related parents. The reason has been well put by Shariati (1996), the founder of first ever genetic counseling centers in Iran: "In more than 90% cases, the chromosomal disorder in the child having Down's syndrome has no relation to the chromosomal condition of the parents, and usually both the parents have a normal chromosomal arrangement as the defect mostly due to mutation..." Almost an equal frequency (10.6%) of primary amenorrhoea was observed in children born to non-consanguineous couples.

Out of the 43 subjects with at least one affected sibling already present in the family, 93% of them were born to consanguineous parents. Thus only 7% of the subjects with one earlier sibling affected were born from non-consanguineous marriages (Table 3). The frequency/chance being almost 13 times more common in the related couples to give birth to an affected child. In other words, the consanguineous parents supporting one genetically abnormal child are almost 13 times more likely to give birth to another affected child as compared to non- consanguineous couples.

In case of the patients with two siblings affected, they all had consanguineous parents, and the only case where 4 siblings in a single family were affected also had parents who were parallel-cousin (Table 3). It is important to note that out of 40 (5%) cases of the consanguineous parents where one or more affected children were already present in the same family, majority was born out of parallel-cousin marriages. In fact, the frequency of occurrence was twice in parallel-cousin when compared with those occurring out of cross-cousin marriages. Interestingly, for unknown reason an equally high frequency was observed when the parents were even distantly related (Table 3). May be these couples were not as distantly related as assumed. It seems that the exact relationship and the level of consanguinity needs to be reexamined.

Consanguinity, more specifically parallel cousin marriages, thus emerges out to be the single most important factor where the family has a history of having children with genetic disorders. This finding contradicts the earlier suggestion that inbreeding reduces the burden of deleterious genes (Rao and Inbaraj 1980).

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DISSERTATION SUMMARY

Cloning and sequence analysis of *Mucor circinelloides* glyceraldehyde-3-phosphate dehydrogenase gene and development of new vector systems for transformation of zygomycetes

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Mucor circinelloides is a filamentous fungi belonging to the Zygomycetes. Its special biochemical, morphological and physiological features established a longstanding interest of both applied and theoretical research. Until now, the transformation systems developed in *Mucor circinelloides* are based on complementation of (*e.g.* amino acid) auxotrophy. In spite of the efficiency, such transformation systems have a serious drawback: a definite stable mutant has to be isolated from each strain before the transformation. Therefore, it seems desirable to establish a transformation system based on a strong native promoter allowing efficient expression of heterologous resistance gene as selection marker.

In the present study a genomic library of *Mucor circinelloides* ATCC 1216b has been constructed in Lambda Fix II vector. The library has an average insert site of 10 kb and covers the genome 12 times. The *M. circinelloides* gene encoding glyceraldehyde-3-phosphate-dehydrogenase (*gpd*) was isolated from this library by hybridization of the recombinant phage clones with a *gpd*-specific gene probe generated by PCR reaction. The complete nucleotide sequence encodes a putative polypeptide chain of 339 amino acids interrupted by 3 introns. The predicted amino acid sequence of this gene shows a high degree of sequence similarity to the GPD proteins from other filamentous fungi. The promoter region containing a consensus TATA and CAAT box and a 298 nucleotid long termination region were also determined (Ács et al. 2002). The predicted amino acid sequence of this gene shows a high degree of sequence similarity to the glyceraldehyde-3-phosphate dehydrogenase proteins from yeast and filamentous fungi (Papp et al. 2003; Vastag et al. 2003).

New transformation systems based on selective drugs have been tested for use in zygomycetes. Transformation vectors have been constructed which contain hygromicin B resistance gene under the control of the promoter of the glyceraldehide-3-phosphate-dehidrogenase (*gpd*) gene from *M. circinelloides*. In contrast to other transformation systems which rely on nutritional auxotrophic markers for the selection of transformants, the combination of a *gpd* promoter sequence and a dominant selectable marker allows the transformation of wild type strains (Nyilasi et al. 2003). Optimal conditions for transformation which increase the sensitivity of these fungi for hygromicin have been worked out.

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DISSERTATION SUMMARY

Small heat-shock proteins can stabilize heat-stressed membranes

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Small heat-shock proteins (sHSPs) are reported to protect (substrate) proteins against irreversible aggregation (van Montfort et al. 2002). However, growing evidence suggests that sHSPs are also involved in the maintenance of membrane integrity during stress, ageing or pathophysiological conditions (Török et al. 2001; Narberhaus 2002).

Our results indicate that sHSPs bind to lipids stabilizing the bilayer liquid-crystalline state affecting both the polar headgroup region and the hydrophobic core (Tsvetkova et al. 2002). Besides, the nature of sHSPs-membrane interactions largely depends on the lipid composition. Specific protein-lipid interactions seem to be operating in membrane reorganization paralleled with increased resistance to stresses (*e.g.* heat, oxidative, etc.).

Photosynthesis is one of the most heat sensitive cellular function in photosynthetic organisms. It is clearly emerging that cell membranes, when exposed to an abrupt increase in temperature, undergo an immediate reorganization through changes in the membrane physical structure (Horváth et al. 1998). In cyanobacteria membrane microdomain reorganization is paralleled with the appearance of highly saturated monoglycosyl diacylglycerol and the association of heat shock protein with the thylakoid.

Non-bilayer forming lipids (MGLDG, MGDG) are of striking importance in HSP17 interaction which shows a remarkable fluidity dependence underscoring the relevance of membrane hydrophobic interior in a protein-induced membrane reorganization.

By assuming that lipid phase of membranes act as cellular thermometer (Vigh et al. 1998) membrane association of sHSPs may constitute a feedback regulation of the expression of stress genes.

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DISSERTATION SUMMARY

Dynamics of long polyunsaturated fatty acids in rat brain

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The brain is one of the organs rich in phospholipids, especially two polyunsaturated fatty acids (PUFA), docosahexaenoic acid (22:6n-3, DHA) and arachidonic acid (20:4n-6, AA). Although DHA is known to play a very important role in many neural functions including learning and memory, its exact function is still unknown (Sastry1985). On the other hand recent evidence attribute similar function to a proper mixture of n-6 to n-3 octadecapolyenoic fatty acids (Yehuda 1998).

Another factor that might be important to consider is the age of the animal. Aging is a physiological process associated with a loss in cognitive faculties and decreased level of DHA (McGahon BM 1999). Despite the generally accepted idea that it is almost impossible to alter brain fatty acid composition of adult rats we showed that they might have a faster turnover in brain polyunsaturated fatty acids than it was believed till this moment. After one month of feeding with lipid-enriched diet, differences at the molecular level in brain phospholipids appeared to be significant.

Finally it is known that PUFA content affects membrane biophysical properties and it was proposed that the beneficial effect on mental functions of this fatty acid mixture realizes at this level. However, it is evident that storing and processing of incoming information is a more complex process and thus cannot be a simple function of membrane physicochemical properties. Investigating the effect of the lipid composition of the diet it was found, that along with alterations in molecular composition of ethanolamine phosphoglycerides, a number of genes are overexpressed in rat brain.

The aim of our studies was to shed some light on the unravelling mechanism through which the long chain polyunsaturated acids exert its important role in brain. For this purpose several feeding experiments were run and fatty acid and molecular composition of brain lipids was determined. The diet consisted in normal rat chow enriched in different oils depending on their composition. Fish oil, perilla and soybean oil were use to have diets rich in DHA, linolenic acid (18:3n-3, LNA) and linoleic acid (18:2n-6, LA), respectively.

Essential fatty acid-sufficient rats of different ages were fed from conception with oils rich either in LNA, the precursor of DHA, eicosapentaenoic (EPA)+DHA, or with a mixture of oils giving a ratio of LA to LNA 8.2 or 4.7.

Phospholipids were extracted and separated depending on their hydrophilic head by thin layer chromatography from brain, cerebellum and hippocampus. Their composition in fatty acids was determined by gas chromatography after transmethylation.

Phosphoglycerides can be divided in three subclasses depending on the nature of the linkage that binds the fatty acid to the sn-1 position in the glycerol backbone. So, one can distinguish diacyl, alkylacyl and alkenylacyl phosphoglycerides. Only PE and PC, preferentially the first one, showed differences at this level, therefore the analysis were focus on this two particular phospholipids. The molecular composition of the three subclasses was determined by high-pressure liquid chromatography.

Gene expression analysis was done by microarray techniques.

We showed that brain fatty acid and molecular species composition, particularly of PE can be modified by a diet depending manner. The major finding is that the response of brain to dietary fatty acids, like LA, LNA and DHA is faster than that was described so far by others. And the same fact was observed for aged rats.

Despite of this alteration in molecular composition in brain membranes, the rats fed on short time bases, did not perform better in Morris water maze test. Therefore, it was concluded that accumulation of DHA alone cannot explain the beneficial effects of long chain polyunsaturated fatty acids on cognitive functions in rats.

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DISSERTATION SUMMARY

The role of phosphatidylglycerol in the photosynthetic electron transport processes

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Thylakoid membranes in chloroplasts and cyanobacterial cells are the sites of the primary processes of oxygenic photosynthesis. The lipid composition of thylakoid membranes is highly conserved and consists of mostly glycolipids and about 10% phosphatidylglycerol (PG) as the only phospholipid. PG is considered to play an important role in the ordered assembly and structural maintenance of the photosynthetic apparatus.

In order to investigate the function of PG in the photosynthetic electron transport processes we studied a *pgsA*$^-$ mutant which was constructed from a cyanobacterial strain, *Synechocystis* PCC 6803. This mutant is defective in PG phos-phate synthase gene, and it is incapable of synthesizing PG and requires PG supplementation for growth. The deprivation of PG from the growth medium decreased the PG content in the mutant cells and it blocked cell growth and led to 50% decrease of oxygen evolving activity on the chlorophyll basis (Hagio et al. 2000).

Artificial quinones which are known to function as efficient electron acceptors for PSII completely inhibited the oxygen evolution of the *pgsA* mutant cells after 5 days of PG deprivation.

Direct evidence for suppression of the electron transfer between Q_A and Q_B was obtained by the thermoluminescence and flash-induced fluorescence measurements of *pgsA* mutant cells (Gombos et al. 2002). The observed effects were reversed if PG was re-added to the cultural medium. These results suggest that PG acts and binds close to the Q_B-binding site of PSII.

Two PG molecules were found at the periphery and one more at the central core of PSI when the PSI reaction center structure resolved at 2,5 Å revealed by Jordan et al. 2001). It has been suggested that the depletion of PG from PSI complexes can cause changes in the function of PSI.

We cultured *pgsA* mutant cells in the PG depleted medium for weeks in order to follow depletion and degradation of PG from PSI complexes. Sixty percent decrease was observed in the activity of PSI by measuring flash-induced absorbance changes at 705 nm after 3 weeks of PG deprivation. At the same time chlorophyll content of the cells decreased to 20% of the initial concentration. PSI activity and the normal chlorophyll content were fully recovered if the cells were grown in the PG-containing medium for a few days.

The observed changes were confirmed by 2-D spectrum/decay-imaging spectroscopy.

The measurement showed significant differences between *pgsA* mutant cells cultured in the presence or absence of PG. The most striking effect was the very low fluorescence at 730 nm in the cells grown in the absence of PG. A broad peak at 730 nm represents long-wavelength absorbing chlorophyll a band typical in PSI reaction center. It can be concluded that PG depleted *pgsA*$^-$ cells cannot form proper PSI reaction center complex at all.

These results were also supported by Deriphat PAGE, a special native gel electrophoretic method (Peter and Thornber 1991). We showed that the amount of the aggregated form of PSI, which is the most efficient form in cyanobacterial energy transfer process, decreased after 3 weeks of PG deprivation. The loss of PSI trimers can be reversed by adding PG again.

We separated PSI monomers from PSI trimers on a preparative HPLC anion-exchange chromatography column. The separated forms were identified by measuring fluorescence emission and excitation spectra of fractions at 77 K, and the proteins were characterized by SDS PAGE.

On the basis of our experiments we concluded that PG is essential for promoting the formation of protein oligomers.

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DISSERTATION SUMMARY

Characterization of the plant anaphase-promoting complex: gene expression and protein-protein interaction studies

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Endoreduplication, the duplication of the genome without division, is a widespread process during the development of various plant organs. It supposes the modification of the cell cycle, G2-M transition needs to be inhibited and DNA-synthetic phase maintained. Investigation of alfalfa nodulation led to the identification of *ccs52*, a cell cycle switch gene encoding a putative regulator of the transition from division to differentiation. Its animal orthologs, members of the Cdh1/fizzy-related family, contribute to the activation of the anaphase-promoting complex (APC).

The anaphase-promoting complex is a cell cycle-regulated ubiquitin-protein ligase whose activity is essential for progression through mitosis. APC is active during mitosis and G1 cell cycle phase; it mediates the ubiquitination of various regulatory proteins, targeting them for degradation by the 26S proteasome. Although APC is a large multiprotein complex (it has at least 12 subunits in *S. cerevisiae*) and its phosphorylation state varies during the cell cycle, its full activation requires the binding of one member of either the fizzy or the fizzy-related family of activator proteins. These activators are thought to confer substrate specificity to the complex by recognizing proteins containing either D- or/and KEN-boxes. Proteins with such motifs (*e.g.* securin, an inhibitor of sister chromatid separation or mitotic cyclins, that have to be degraded for the inactivation of cyclin-dependent kinases) are key regulators of mitotic events.

Despite the recent development in understanding APC function and regulation many questions remain unanswered. This is particularly true for the plant APC which is still unexplored.

The completed Arabidopsis genome allows to identify homologs of almost all of vertebrate APC subunits. In order to give a first characterization of the plant complex and to get an insight into its interaction with Ccs52 proteins, full length cDNAs of certain of the predicted subunits were isolated from an Arabidopsis cDNA library. These were the TPR repeats containing CDC23, CDC16, CDC27.2 as well as the CDC27/HOBBIT; APC2 that contains a C-terminal cullin-homology domain, the Doc domain protein APC10, CDC26 and the APC11 RING-H2 finger protein.

In attempt to define the molecular organization of the plant APC, we tested all possible interactions among the cloned subunits as well as their association with the members of the Arabidopsis Ccs52 family by yeast-two hybrid analysis. Strong interaction was observed between APC2 and APC11 and the yeast two-hybrid result was verified by co-immunoprecipitation of the epitope-tagged proteins.

To study the role of these genes during the cell cycle, Arabidopsis cell suspension was synchronized with aphidicholin. The APC-subunit coding genes have constant expression level during the cell cycle, which suggests the importance of posttranslational modifications in the regulation of the activity of the complex. In contrast, the expression of the three *ccs52* genes shows a strong cell cycle regulation, and this coincides well with the pattern of the corresponding substrates.

Although the structure of the APC may depend on multiple protein-protein interactions, our results demostrate a pairwise interaction between the subunits APC2 and APC11. The gene expression studies support that the transcriptional regulation of APC function is mainly based on cell cycle phase-specific expression of the APC activator genes.

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DISSERTATION SUMMARY

Higher order chromatin structure and gene regulation in *Drosophila melanogaster*

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Segmental identity in Drosophila is determined by two clusters of homeotic genes, the *Antennapedia-* (ANT-C; Kaufman et al. 1990) and the *bithorax-* (BX-C; Lewis 1978) complexes. The complex expression pattern of the BX-C genes is due to the action of nine parasegment-specific *cis*-regulatory domains (Duncan 1987). The activity patterns of these *cis*-regulatory regions are set early in development by protein products of segmentation genes (Shimell et al. 1994). By midembryogenesis, when the products of the segmentation genes disappear, the regulation of the homeotic genes switches to a maintenance mode that preserves the initial pattern of activity through the remainder of development (Paro 1993). Maintenance of the inactive state requires the action of the Polycomb-group (PcG) of proteins. By contrast, the *trithorax*-group (trxG) of genes is responsible for maintaining the active state of homeotic genes. PcG proteins function cooperatively and form multimeric repressor complexes (Shao et al. 1999), which are tethered to the DNA at sequences called Polycomb Response Elements (PRE). The antagonistic activities of trxG and PcG proteins involve modulation of chromatin structure. A deletion that removes the *Frontabdominal-7 cis*-regulatory region (*Fab-7*) removes a domain boundary element (transforming parasegment 11 into parasegment 12; Gyurkovics et al. 1990) and a silencer element, the *iab-7* PRE (Hagstrom et al. 1997). Transgenic lines containing *iab-7* PRE fragments show pairing-sensitive silencing of the *miniwhite* reporter gene: the eye color of transgenic flies is lighter in homozygous than in heterozygous conditions. This silencing effect is weakend by introducing a PcG mutation, while it is strengthened in a trxG mutant background.

We used two transgenic constructs containing two different *iab-7* PRE fragments for large scale screens to identify previously unknown PcG and trxG genes. Lighteners of eye color were supposed to be caused by mutations in trxG-, while darkeners in PcG genes. We found several mutations in previously characterized, and yet uncharacterized PcG and trxG genes, indicating that our screening method is sensitive enough to identify new members of these groups of genes. After establishing the complementation groups we tested the identified mutations in different *in situ* systems, and in transgenic systems. We have proved that not all members of the PcG are necessary for silencing on a given PRE. We have also shown that the effect of some PcG genes is specific to a given PRE. The fact, we could also identify lighteners of the eye color using a PRE containing transgene indicating, that a TRE-like (Trithorax Response Element) is overlapping or located very closed to the *iab-7* PRE. We could also demonstrate a regulatory network between members of the PcG and trxG genes. Some mutants (*gpp* and *bon*) showed controversial phenotypes: Polycomb-type homeotic phenotype while lightening of the eye color, or trithorax-type homeotic phenotype while darkening the eye color of the transgenic construct. These gain-of-funtion mutants were reverted using X-rays. The revertants were cytologically analysed for identifying the locus, and used for genetic experiments to compare the gain-of-function and loss-of-function phenotypes to further characterize the function of the given genes. Recently we designed four new transgenic constructs carrying different fragments of the *iab-7* PRE cloned between Flp recombinase target sites. FRTs are used to select lines showing pairing-sensitive silencing without the transgenic PRE being involved in any interactions of endogenic PRE sequences. These constructs were injected to embryos. The establishing of transgenic lines is in progress.

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DISSERTATION SUMMARY

Use of pseudorabies virus to delineate plastic neuronal circuits in the brain: opportunities and limitations, and possible background

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It has earlier been demonstrated that strain Bartha (PRV-Ba), an attenuated pseudorabies virus (PRV) live vaccine virus, is suitable as a retrograde tracing tool for revealing neural circuitries. Although fewer studies have employed intracerebral injections of the virus, available evidence suggests that this experimental approach is also an effective means of defining multisynaptic circuits. We tested whether this method based on the use of this virus is sensitive enough to detect fine plastic changes induced in the central nervous system (CNS).

A number of publications have demonstrated that oestrogen induces plastic alterations in the synaptic connections in the CNS. We examined that neuronal infectivity and the spreading of PRV through the synapses in the CNS are influenced by the oestrogen levels. The arcuate nucleus and the subfornical organ were chosen as models for analysis; the neurons in both structures possess oestrogen receptors and are mutually connected. Our results demonstrate that transneuronal PRV labelling depends on the effects of oestrogen on certain CNS structures and connections (Horváth et al. 2002).

In adult mammals, the somatotopic representation map of the muscle system in the motor cortex is not stable, but may be modified within hours to days after peripheral facial nerve injury. In particular, we have previously demonstrated an facial nerve injury (N7x)-induced early disinhibition of the commissural connections between the primary motor cortices (MIs) by intracortical microstimulation of the facial muscle representation field. In our second experiment, unilateral N7x was found to influence the transcallosal spread of PRV-Ba from the affected (left) primary motor cortex (MI) to the contralateral MI of rats (Horváth et al.).

To explain these results, it should be taken into account that the entry of alpha herpes viruses into the cells usually requires multiple interactions between the viral envelope and the cell surface proteins. At least two groups (HSPGs and nectins) of these cell surface glycoproteins are known to play roles in these processes (Mettenleiter 2000). It should also be taken into account that HSPGs and nectins participate in the development and plasticity in adulthood of tissues of neuroepithelial origin (Mizoguhi et al. 2002; Rauvala and Peng 1997). We have shown here that oestrogen level and N7x does not affect the entry of PRV, but increases the efficiency of its cell-to-cell spread. Thus, the infection pattern does not appear to be related to cellular components (HSPGs) involved in the attachment of the virus, but rather to cellular components located at the synaptic region of the membrane of post-synaptic neurons.

Nectin-1 and nectin-2, components of a novel cell-cell adhesion system, and localized within the cadherin-catenin system at cell-cell adhesive junctions, have been shown to play important parts in synapse formation (Mizoguhi et al. 2002). In ep-ithelial cells, dissociation of the cell junctions releases nectin-1 to serve more efficiently as an entry receptor. Sakisaka and coworkers (2001) have reported that the interaction of nectin-1a with afadin does not affect the entry of HSV-1, but increases the efficiency of the cell-cell spread of that virus. The mechanism by which oestrogen level and N7x increases the efficiency of cell-cell spread of PRV in the cortical network *in vivo* remains to be elucidated, but one possible explanation will be discussed.

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DISSERTATION SUMMARY

Ceramide mediated apoptotic pathway is involved in galectin-1 induced apoptosis in Jurkat T lymphocytes

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Galectin-1, a member of the S-type mammalian lectin family is characterized by high affinity for β-galactoside on complex glycoconjugates and by a highly conserved carbohydrate recognition domain (CRD). Galectin-1 induces apoptosis of immature cortical thymocytes, activated peripheral T lymphocytes and T leukemia cell lines. According to this function, galectin-1 is a potential valuable therapeutic agent in autoimmune and inflammatory diseases. In spite of the increasing interest, the molecular mechanism of the apoptotic process is not well understood yet.

In the present study, we examined the possible involvement of protein kinase C (PKC) and ceramide in apoptosis induced by galectin-1. Recently, we showed that $p56^{lck}$, the non-receptor tyrosine kinase is involved in the galectin-1 triggered apoptosis. The literature data indicated that ceramide, an apoptotic lipid second messenger also dependent on the presence of functional lck. Upon this finding we have been analysing whether galectin-1 acts through the ceramide mediated apoptotic pathway. The latter was analysed by exogenously added C6-ceramide, as a model. We found that: 1) Phorbol 12,13-dibutyrate (PDBu) inhibited the galectin-1 induced apoptosis by 50-100%. Since PDBu directly activates PKC, we suggest that PKC activation counteracts galectin-1-mediated apoptosis similarly to that induced by ceramide. 2) A caspase-8 inhibitor did not affect the apoptosis either induced by galectin-1 or ceramide. In order to clearly determine whether caspase-8 is requisite for galectin-1 mediated apoptosis we compared the sensitivity of the wild type and the caspase-8 deficient ($J\text{-}C8^{mut}$) Jurkat cells. Both cell lines were similarly sensitive to galectin-1 and ceramide but the caspase 8 mutant was resistant to apoptosis initiated via the TNF receptor. 3. To gain a direct evidence for the role of the ceramide pathway in this process we used fumonisin B1 a specific inhibitor of ceramide synthesis. This drug did not inhibit the effect of galectin-1 indicating that production of ceramide upon galectin-1 stimulation did not occur through the synthetic route. Further we studied whether the catabolic production of ceramide is involved in galectin-1 induced apoptosis. For this purpose we used another inhibitor of ceramide-mediated apoptosis, sphingosine-1 phosphate (S1P). S1P inhibited the galectin-1 or ceramide induced apoptosis similarly. Thus, S1P, likely generated via PKC-mediated activation of sphingosine kinase, downregulates the galectin-1 apoptotic pathway. Taken together, our results strongly suggest that galectin-1 induced apoptosis is mediated by the intracellular ceramide level, leading to the activation of a caspase cascade.

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DISSERTATION SUMMARY

Role of mobile introns in mtDNA polymorphisms of imperfect black *Aspergilli*

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Aspergillus niger and *Aspergillus tubingensis* belong to an imperfect group of genus *Aspergillus*, section *Nigri* (Gams et al. 1985). Both species exhibit intraspecific polymorphism especially regard to their mtDNA RFLP patterns. *A. niger* and *A. tubingensis* consist of several mtDNA RFLP groups (1a-1e groups of *A. niger*, and 2a-2f groups of *A. tubingensis*; Varga et al. 1994). The physical maps of the *A. niger* strain representing mtDNA RFLP type 1a and the *A. tubingensis* harbouring mtDNA RFLP type 2b were constructed by *Eco*RV, *Eco*RI, *Bgl*II and *Hind*III restriction enzymes. The sizes of the mtDNAs of 1a and 2b strains were 31.26 kb, and 33.09 kb, respectively. The 1.5 kb size difference observed between mtDNAs of *A. niger* and *A. tubingensis* were principally attributed to the altered intron content of their *cox*1 gene. The 2728 bp *cox*1 gene of the *A. niger* harbours one group I intron (1025 bp), its ORF codes a LAGLIDADG type endonuclease. The 5058 bp *cox*1 gene of the *A. tubingensis* possesses three group I introns (1148 bp, 1126 bp and 1084 bp, respectively) their ORFs coding for LAGLIDADG type endonucleases, too. The second intron of *A. tubingensis* is identical with the intron of *A. niger* in their sequences and insertion sites, however intron-flanking exonal sequences differ in some nucleotides. The first intron of *cox*1 of *A. tubingensis* exhibits high homology to the second intron of *cox*1 of the *A. nidulans*, however, the third *cox*1 intron of the *A. tubingensis* does not show homology to any known mitochondrial intron. To study the mobility of the first and the third *cox*1 introns of *A. tubingensis*, interspecific mitochondrial transmission experiments were performed by protoplast fusion between a mitochondrial oligomycin resistant, *A. niger* 1a strain and an oligomycin sensitive *A. tubingensis* 2b strain with selectable nuclear markers (Kevei et al. 1997). Following protoplast fusion the progeny were recovered selecting for the *A. tubingensis* nuclei and for the oligomycin resistant *A. niger* mitochondria. The 50 recovered resistant progeny were analysed, they represented six RFLP types differed from those of the parents. To study of the rearrangements of the mtDNA all types of these recombinant and the parental mtDNAs were mapped with four restriction enzymes and compared. Arrangement of the restriction sites of the two parental mtDNAs showed some differences (1.5 kb in their sizes). The most of the different restriction sites did not result in detectable size-differences except the ones situated in *cox*1 gene. Sequence analysis of the *cox*1 revealed that two additional group I introns were present in the *cox*1 of the recipient as compared to the donor. Using intron specific primers we proved, that all types of recombinant-like mtDNAs contain the same introns in the same positions as those of in the recipient. All progeny inherited the resistant mtDNA of the *A. niger*, but the *cox*1 gene was always invaded by the first and the third *cox*1 introns of *A. tubingensis*.

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DISSERTATION SUMMARY

Expression of matrilins in embryo and newborn mice

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Matrilins form a structurally and evolutionarily related group of extracellular matrix proteins. The matrilin family at present has four members, all have the same structure, build up of von Willebrand factor type-A domains, epidermal growth factor-like modules and a coiled coil α-helical module. The prototype is matrilin-1, previously called cartilage matrix protein (CMP). Matrilin-1 and -3 are mostly found in cartilage, matrilin-2 and -4 show a wider tissue distribution. Recently we cloned matrilin-2 cDNA and determined the expression pattern of the gene in adult mice. In order to gain a better understanding of the function of the protein, and to see a potential role in development, we monitored the expression of matrilin-2 during mouse embryo development and in the newborn animal.

RNA and tissue samples were collected from mouse embryos to monitor changes in the expression of the matrilin-2 gene. Matrilin-2 mRNA was detectable by RT-PCR already at the age of E7.5, and the amount of it increased during embryo development. Protein deposition was examined by indirect immunofluorescence or immunohistochemistry in cryostat sections. In 7.5 day embryos matrilin-2 was detectable in large amount in the ectoplacental cone and the decidua and in extraembryonic tissue. At day 9.5 the protein was seen in the basal lamina of the somite epithelium, and in the primordial meninges around the brain vesicles. At day 11.5 deposition of the protein can be observed in the notochord, the wall of dorsal aorta, in the zones of vertebral body primordia and in the developing dorsal root ganglia. Matrilin-4 is more readily detectable in these places. At day 13.5 all the skeletal elements are stained for matrilin-2. In the developing nervous system, the brain and the medulla spinalis are not labeled for matrilin-2, except for the pia matter, the basal lamina of ependymal cells and the choroid plexus. However, the cranial nerves and ganglia, the dorsal root ganglia and the spinal nerves and also the sympathetic trunk show matrilin-2 deposition in the peri- and epineurium. To identify the expressing cell type, nonradioactive *in situ* hybridisation was performed. Expression of the matrilin-2 gene was clearly found in the sensory neurons of the dorsal root ganglia. During eye development, matrilin-2 accumulates almost as early as the lens is formed. At day 11.5 the protein was observed in the lens capsule. It is also visible in sclera and several layers of the optic cup. The corneal stroma, however, which contains abundant quantity of matrilin-4, does not show accumulation of matrilin-2, suggestive of different interacting collagen partners for the two matrilins.

One of the further aims of my study was to compare the expression pattern of matrilins in the developing skeleton using the same techniques. Matrilin-1 is detectable earlier than matrilin-3, but by newborn age expression domain of matrilin-3 becomes broader including the zone of reserve chondrocytes and osteoblasts. Matrilin-2 is less abundant in cartilage with a relative maximum in the zone of the hypertrophic cells, but detectable in large amount at the articular surface, perichondrium, periosteum and menisci (Segat et al. 2000).

The matrilin-2 gene expression was monitored in various cell lines. Both the human and mouse genes are transcribed from two promoters. The upstream, housekeeping-like promoter is active in all cell types tested, while the downstream, TATA-like promoter functions only in embryonic fibroblast and in certain cell lines (Mátés et al. 2002).

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DISSERTATION SUMMARY

Calmodulin gene expression in central nervous system areas of low calmodulin mRNA abundance

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The calmodulin (CaM) protein plays a complex role in the regulation of the different cytoplasmic processes in general, and also (because of its paramount role in the neuronal signalization processes) in the nervous system as a whole in particular. While this protein is often present in medium-to-high concentrations in the nervous tissue, there are other regions (the spinal cord and the retina) that contain CaM only in minute quantities, and the amounts of the different CaM transcripts in these regions are also low. Our knowledge on the CaM gene expression in the different cell types of the spinal cord and the retina is sparse, mainly in consequence of their low CaM mRNA contents. Thus, during the initial stage of our research, we developed a sensitive color in situ hybridization method (Kovacs and Gulya 2001), employing a slightly alkaline pH (pH 8.0) in the hybridization mixture, which is sensitive enough to detect low-abundance CaM transcripts in areas of the central nervous system (the white matter of the spinal cord and the retina) that may also have high lipid contents (the white matter areas of the spinal cord).

On the basis of their morphology, we detected two distinct cell types that express CaM in the white matter area of the rat spinal cord (Palfi et al. 2002; Kovacs and Gulya 2002). The medium-sized, astrocyte-like cells residing mainly in the dorsal column of the white matter displayed differential CaM expression: the CaM I mRNA content was highest, followed by the CaM III and CaM II contents in these cells. The CaM gene expression of the oligodendrocytes (in both the dorsal and the lateral columns) was less differentiated, although the CaM I mRNA content was slightly higher than that of the others. Our results indicate that 1) the CaM expression profile of the spinal cord is richer and more complex than previously thought on the basis of conventional radioactive in situ hybridization techniques, and 2) when a method that is sufficiently sensitive was used, more cell types could be demonstrated to express CaM mRNAs; thus, in spite of their lower CaM expression and their lipid-rich environment, glial cells could also be visualized.

The retina is a tissue with low lipid content, where the conventional in situ hybridization techniques with relatively low sensitivity did not detect any CaM-expressing cells, in spite of the fact that the presence of the protein was previously demonstrated by immunohistochemical techniques. Our sensitive in situ hybridization technique revealed the presence of CaM mRNA populations in the adult rat retina, and we concluded that the expression levels of the different CaM genes were almost identical (Kovacs and Gulya 2003). Although the layer-specific distributions of these genes are strikingly similar, there are major differences in CaM expression within the different retinal layers. The strongest signals for all CaM mRNAs were demonstrated in the ganglion cell layer and the inner nuclear layer. Intermediate signal intensities for all CaM genes were detected in the inner and outer plexiform layers, within the vicinity of the outer limiting membrane and in the pigment epithelium. Very low specific signals were characteristic in the outer nuclear layer and the photoreceptor inner segment layer, while no specific hybridization signal was observed in the photoreceptor outer segment layer.

In summary, our sensitive in situ hybridization technique was able to detect low-abundance CaM transcripts in the white matter of the rat spinal cord and the neural retinal of the adult rat (Kovacs 2003). Our results contribute to a better understanding of the functional role(s) of the CaM protein and also of the CaM gene expression in these regions.

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DISSERTATION SUMMARY

Studies on the signal transduction cascades responsible for the control of the expression of NiFe hydrogenases and photosynthetic apparatus in purple sulfur photosynthetic bacteria

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The photosynthesis and hydrogen metabolism play important role in the energy metabolism of photosynthetic bacteria. If they are energetically linked, the expression of their components should be regulated by common factors. A pigment mutant strain of the purple sulfur photosynthetic bacterium, *Thiocapsa roseopersicina* BBS was isolated by plasposon mutagenesis. About 19 *orf*-s, most of which are thought to be genes involved in the biosynthesis of carotenoids, bacteriochlorophyll and photosynthetic reaction centre were identified surrounding the plasposon in a 22 kb long chromosomal locus. The carotenoid biosynthetic genes, *crtDC* and *crtE* genes were shown to be regulated by oxygen, and the role of CrtJ in aerobic repression was suggested (Kovács et al. 2003).

T. roseopersicina harbors two membrane bound [NiFe] hydrogenases (HupSL, HydSL). The two enzymes differ in their stability in the presence of oxygen, heat and proteases (Kovács et al. 2002). The organization of the *hyn* operon is extraordinary, since two additional *orf*-s (*isp1* and *isp2*) separates the structural genes: *hynS* and *hynL* (Dahl et al. 1999). The maturation of these enzymes requires several accessory proteins, which are involved in e.g. metal incorporation, formation of the active centre, the proteolytic cleavage of the large subunit (Fodor et al. 2001; Maróti et al. 2003).

Generally the expression of HupSL type regulation is controlled via a H_2 sensing system. We identified genes coding for the hydrogen sensor (HupUV) and the sensory kinase (HupT) of this signal transduction cascade. In spite of the presence of these genes, the expression of *hupSL* was apparently not effected by H_2, as indicated by hydrogenase activity measurements and *lacZ* fusion constructs, but repressed by traces of oxygen. The expression of the *hydSL* was also shown to be enhanced in the absence of oxygen. Upstream from the determined promoters a region was identified as an essential *cis* element for this anaerobic activation. The regulation of the *hyd* operon by O_2 could be observed in *Escherichia coli* and *Rhodobacter capsultus*, as well. The role of the FNR, but not the ArcAB or RegAB systems in the anaerob activation was demonstrated in *E. coli*, and in *R. capsulatus*. The comparison of these regulation styles will be discussed.

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DISSERTATION SUMMARY

Subcellular information processing: placement and effect of GABAergic synapses, gap junctions and hyperpolarization-activated ion channels on cortical neurons

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Synaptic integration and information processing is highly affected by the placement of synaptic inputs on the somato-dendritic surface of cortical neurons. Distinct interneuron populations innervate perisomatic and dendritic regions of cortical cells and have significant role in governing neuronal activity at behaviorally relevant frequencies.

Perisomatically terminating GABAergic inputs are effective in timing postsynaptic action potentials, and basket cells synchronize each other via gap junctions combined with GABAergic synapses at γ frequency (Tamas et al. 2000).

Regular spiking nonpyramidal cells (RSNPs) innervate dendritic shafts and spines and occasionally somata. Combined GABAergic and gap junctional connections produce synchronous activity of the coupled RSNPs, however strong electrical coupling can also synchronize presynaptic and postsynaptic activity at β and γ frequency (Szabadics et al. 2001).

Inhibition in the cerebral cortex consists of fast $GABA_A$ and slow $GABA_B$ receptor mediated inhibitory postsynaptic potentials (IPSPs). Most neuron classes elicit IPSPs through $GABA_A$ receptors, but possible distinct sources of slow inhibition remained unknown. We identified a class of GABAergic interneuron, the neurogliaform cells, that in contrast to other GABAerg cells, elicited combined $GABA_A$ and $GABA_B$ receptor mediated responses and predominantly targeted dendritic spines of pyramidal neurons. Slow inhibition evoked by a distinct interneuron in spatially restricted postsynaptic compartments could locally and selectively modulate cortical excitability (Tamas et al. 2003).

Hyperpolarizing the dendritic membrane, IPSPs evoked by GABAergic inputs can activate hyperpolarization-activated cation channels that can influence the summation of synaptic inputs and determine how sub-and supratreshold events propagate to soma. However, the functional role of an ion-channel depends, to a large extent, on its location and density on the surface of nerve cells. Using high-resolution immunolocalization we determined the subcellular distribution of the hyperpolarization-activated and cyclic-nucleotide-gated channel subunit 1 (HCN1). Quantitative comparison of immunogold densities showed a domain-, distance- and subcellular compartment dependent distribution of HCN1, revealing the complexity in the cell surface distribution of a voltage-gated ion-channel, and predict its role in increasing the computational power of single neurons via subcellular domain and input specific mechanisms (Lorincz et al. 2002).

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DISSERTATION SUMMARY

Adaptation of synthetic oligonucleotide-based inhibition in plant systems

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Antisense oligonucleotides have gained an ever-increasing significance during the last few years. Beyond a therapeutic application, antisense oligonucleotides have proven to be a very useful research tool in the molecular level analysis of life processes, as it is possible to selectively decrease the production of the targeted protein via sequence-specific inhibition.

The aim of this research was to set up an experimental system that enables us to trace the slightest change in the activity of the reporter gene – the luciferase of the firefly, *Photinus pyralis*. First of all, the cellular system and the factors influencing gene expression were optimized. The oligonucleotides exhibited the strongest effect after overnight incubation following transfection.

Our strategy for selecting an oligonucleotide was to choose some functional element of gene expression, such as an ATG-region. The next level of selection was searching for a single stranded region of the mRNA that is accessible for the oligonucleotides. This was optimized with the use of computer modelling.

The following steps were used in designing the oligonucleotides:

First of all, suitable genes were selected (EMBL GenBank database). The applied marker genes were firefly luciferase and GUS. Afterwards, the sequences were sent to a server (mfold 2.3), which calculates the three-dimensional structures of the mRNA. Then matches were located among the open structures, and sections of 18-21 base pairs, suitable for antisense oligonucleotide application were selected. To ensure specificity of the oligonucleotides, homologue sequences were excluded (BLAST 2.0). Next, the T_m points were optimized to physiological environment. Loop and self-complementary sequences were discarded. To increase stability, we used chemically modified oligonucleotides. However, as thioesther bonds are toxic beyond a certain threshold, it was necessary to optimize the number of such bonds within the molecules. In the application step, the uptake of the molecules was first checked, and then measurements were made to investigate the inhibitory effect on the marker genes.

We have found that antisense oligonucleotide-based inhibition is applicable to plants; however, the efficiency of inhibition is somewhat lower as compared to other systems.

Since antisense oligonucleotides exert their inhibitory effect specifically even on the level of the organism, therefore we have attempted to apply the technique to intact plants. Our results show that the inhibition is inducible, but again with less efficiency.

Our other approach gene silencing was through the use of small inhibitory RNAs (siRNAs), which were synthesized artificially with the Expedite system (Applied Biosystem).

RNA interference was discovered a few years ago, and as of yet it has never been applied in plant systems. si RNAs have sense and antisense strands of about 21 nucleotides that form 19 base pairs to leave overhangs of two nucleotides at each 3' end. A double stranded RNAs matching a gene sequence is synthesized in vitro and introduced into a cell. si RNA are thought to provide the sequence information that allows a trigger-specific mRNA to be targeted for degradation.

The non specific pathway is triggered by dsRNA of any sequence as long as it is at least 30 base pairs long. Currently the adaptation of the protocols to plant systems is being tested, simultaneously on protoplasts, cell suspensions and intact organisms.

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DISSERTATION SUMMARY

Learning to say NO...

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Nitric oxide (NO) is the smallest, lightest molecule – and the first gas – known to act as a biological messenger in animals. It is involved in diverse signal transduction pathways controlling smooth muscle tone, responses to infection, apoptosis, cell proliferation as well as fertilization. Recently, the role of nitric oxide in plants has received much attention. It has been alrady demonstrated that plants not only respond to atmospheric NO, but also possess the capacity to produce nitric oxide enzymatically. Initial investigations of NO functions suggested that plants use NO as signaling molecule via pathways remarkably similar to those found in mammals.

Mounting evidences support the hypothesis that NO is a novel effctor of plant growth, development and defense responses. An excellent experimental system to provide further proofs of the involvement of NO in the regulation of plant development is somatic embryogenesis.

In alfalfa, the homogenous population of leaf protoplasts can be induced to form embryogenic cells with reliable synchrony. To find out the role of NO during the reactivation of somatic plant cell protoplasts were cultured in the presence of a NO donor, sodium nitroprusside (SNP) and/or an inhibitor, NG-monomethyl-L-arginine (L-NMMA). Cell morphology and division parameters have been affected by both compounds indicating a role of NO in these processes. Our results suggest, that NO is required for the dedifferentiation process rather than for cell cycle progression and may affect the acquisition of the embryogenic cell fate.

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DISSERTATION SUMMARY

Development of a general strategy to obtain stable transgenic plants in non-embryogenic lines using the *Agrobacterium tumefaciens* plant transformation method

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The aim of our group is to identify, isolate and analyse plant genes required for symbiotic nitrogen fixation. We used the map based cloning strategy to achieve these goals. After genetic mapping of a Nod$^-$ mutation in tetraploid alfalfa, our group carried out the chromosome walking and sequenced the BAC clones covering the NOD region. In this way we could identify a candidate gene coding for a receptor-like protein kinase which carried mutation in the Nod$^-$ alfalfa plant and also in four mutant alleles of *Medicago truncatula* and two mutant alleles of *Pisum sativum* in which the mutation was mapped to the same region (dmi2 and sym19 mutants).

My responsability in our group was to carry out the plant transformation experiments including (A) the complementation of the Nod$^-$ plants and (B) *the in vivo* characterization of the gene and the protein.

(A) We performed the complementation of *Medicago sativa* and *Medicago truncatula* Nod$^-$ plants via *A. tumefaciens* and *A. rhizogenes* transformation method respectively, with the wild type NORK (Nodule Receptor Kinase) gene.

Since the Nod$^-$ alfalfa line is not embryogenic we made a cross with the highly embryogenic Regen S line and in the F1 population we identified two highly embryogenic individuals. For the complementation we used two approaches: (1) identification and transformation of embryogenic Nod$^-$ plants in the F2 population obtained after self-pollination of the embryogenic F1s and (2) after transformation the F1 individuals, self-pollination of transformants and identification of individuals that are homozygous for the mutant allele and carry the transgene.

(1) We identified 15 Nod$^-$ individuals and found one embryogenic Nod$^-$ plant that we transformed with the NORK gene in order to rescue the mutant phenotype. These transgenic plants are developing now and will be analysed for the presence of transgene and for the complementation. (the embryo formation period was prolonged to nine months in the embryogenic test).

(2) We transformed the F1 individuals via *A. tumefaciens* using five different constructs carrying the NORK gene and we obtained transgenic plants with three of them. The transgenic plants were self-pollinated and the individuals of the F2 populations were genotyped for a PCR-based marker closely linked to the NORK gene. Eight individuals of the 622 plants tested were homozygous for the mutant allele. Seven of these plants developed nodules on their roots. Southern blot analysis was performed to confirm that the mutant NORK allele was homozygous in these plants. It was proved that 7 individuals of the 8 candidates were homozygous for the mutant allele and six carried the transgene which complemented the mutant phenotype in all of the cases. In this way we could develop a general strategy to obtain stable transgenic plants in non-embryogenic lines using the *Agrobaterium tumefaciens* plant transformation method. We also transformed Nod$^-$ *M. truncatula* (R38 and TR25) plants via *Agrobacterium rhizogenes* (Arqua strain) using pCAMBIA plant transformation vector carrying the NORK gene. Four plants out of ten carrying transgenic roots were complemented (they have root nodules with bacteroids after infection with *Sinorhizobium meliloti*).

(B) The *in vivo* characterization of the NORK gene and the protein: (a) the temporal regulation of the gene (promoter-GUS fusions). We obtained transgenic *M. truncatula* plants (by *A. rhizogenes* plant transformation) carrying NORK promoter fused to the GUS reporter gene. According to these experiments we can conclude that NORK promoter is a constitutive one, being expressed in the root except the root tip, while to the 35S promoter, used as a control, is expressed in all the tissues of the root. Microscopic studies are necessary to specify in which tissues and cells the NORK gene is expressed. (b) the effect of the overexpression of the gene (35S promoter versus own promoter-NORK cDNA). We obtained transgenic *M. truncatula* plants carrying the NORK cDNA driven by 35S promoter and its own promoter (NORK promoter) but we could not achieve complementation with these constructs.

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DISSERTATION SUMMARY

The enrichment of the genetic map of alfalfa (*Medicago sativa*) and its comparison with other Fabaceae and *Arabidopsis thaliana* genetic maps

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Alfalfa (*Medicago sativa)* is one of the most important cultivated forage legume in the world. The high nutrition level and the capacity to estabilish symbiotic nitrogen fixing interaction with the *Rhizobium meliloti* made alfalfa an important subject for molecular genetic studies. To analyze the genes involved in the control of the nitrogen fixing pathway it was important to construct a detailed genetic map of alfalfa. In our group at the begining of the '90s, a basic genetic map was constructed for alfalfa using 98 RFLP, RAPD, isozyme and morphological markers which could be ordered in eight linkage groups (LG; Kiss et al. 1993). Later an improved version of the genetic map was made on the basis of 1800 PCR-based (CAPS, RAPD, SSCP), RFLP and isozyme markers (Kaló et al. 2000).

The aim of our work was to increase the number of markers with known function on the genetic map of alfalfa and using this information to compare this map with the genetic map of other species from the Fabaceae family and model plant *Arabidopsis thaliana*. The intron-targeting method was used to design new primers using cDNAs of the model legume *M. truncatula* aligned to *A. thaliana* genomic sequences. Since intron sequences are less conserved during evolution by amplification of these segments there is a better chance to detect length polymorphism. Following PCR amplification agarose gel electrophoresis was made to detect length polymorphism or dominant inheritance. The non polymorphic PCR fragments in agarose gels were subjected for further analysis to detect polymophism; either SSCP experiments were performed using polyacrylamid gel electrophoresis or the sequences of the amplified products were determined for the two alleles. In the case of amplification products where no polymorphism could be detected with the above mentioned methods, the sequence of the fragments were determined and possible CAPS sites were identified. The new markers were positioned on the 8 LGs of alfalfa by using the colormap method developed in our laboratory (Kiss et al. 1998). The genetic map of alfalfa was then compared with three different genetic maps of the model legume *M. truncatula*, *Pisum sativum* and *Arabidopsis thaliana*.

The comparison of the genetic maps of the two *Medicago* species (the maps of one *M. sativa* and 3 *M. truncatula* population) was made on the basis of 157 markers with known function or sequence. As expected the alignment of the four genetic maps shows a high degree of conservation in marker order. The few detected differences were mostly due to distinct loci number of some markers. One major difference could be observed concerning the position of the rDNA location. On the genetic map of *M. truncatula* the rDNA marker could be found on the LG5 while on alfalfa is located on the LG6.

M. sativa and *P. sativum* are two related species both belonging to the Fabaceae family. The genetic map of pea contains seven LGs. By comparing the genetic maps of pea and alfalfa a high degree of synteny in the marker order could be detected. Four LGs could be completely aligned to the corresponding alfalfa LGs while in the case of three LGs cromosomal rearrangements could be seen. The information obtained from the comparison of these two genetic maps can be used to enhance the genome analysis of pea which is hindered by its big genome size.

In order to test the degree of synteny between unrelated species the genetic map of alfalfa was compared with that of the *A. thaliana*. For this comparison we selected mapping data for *A. thaliana* from the official webpage www.arabidopsis.org. For a reasonable comparison 268 low copy number ortholog genes were used for our work. From the comparison of the two maps we concluded that no macrosynteny could be detected between the marker order of the genetic map of alfalfa and *A. thaliana*.

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DISSERTATION SUMMARY

Structural and fuctional study of the Drosophila *melanogaster* 19S regulatory complex

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In addition to transcription and translation, regulated degradation plays also an important role in the determination of the halflife of proteins. In eukariotic organisms some of the cytosolic regulator proteins are degraded by the ubiquitin-proteosome pathway. This pathway consists of the ubiquitin ligase system and the 26S proteasome. The components of the ubiquitin ligase system are the ubiqutin polypeptide (Ub), the ubiqitin activatig enzyme (E1), the ubiquitin conjugating enzymes (E2) and the ubiquitin ligase enzymes (E3). This system attaches a multiubiquitin chain covalently to the proteins before degradation. The 26S proteasome is a large multiprotein complex made of two different subcomplexes called 20S core particulum and 19S regulatory complex.The 20S core particulum is a barrelshaped ATP independent aspecific protease. The main role of the 19S regulatory complex is to ensure specificity to the aspecific 20S core protease complex towards multiubiquitinated proteins. The 19S regulatory complex can recognize the substrate proteins multiubiqitinated by the ubiquitin ligase system, unfold and feed them into the 20S core particulum's central cave, where the hydrolysis occurs.

Comparing the immunoblots of one dimensional denaturing polyacrylamide gel electrophoresis (1D-SDS-PAGE) with the immonoblots of the isoelectric focusing-denaturating polyacrylamide gel electrophoresis (2D-IEF-SDS-PAGE), we noticed that some of the antibodies recognising a distinct band on the 1D-SDS-PAGE blots, did not recognise any spot on the 2D-IEF-SDS-PAGE blots. This finding suggested that the former determinations of the 19S regulatory complex subunit composition based on 2D-IEF-SDS-PAGE systems were incomplete. To fully complement this subunit composition we adopted a principaly different gel electrophoresis system called two dimensional 16-BAC- SDS denaturating polyacrylamide gel electrophoresis (2D-16BAC-SDS-PAGE), which reveals all the subunits of the 19S regulatory complex (Hölzl et al. 2000). Using immunoblotting and MALDI aminoacid sequencing, we determined the full subunit composition of the 19S regulatory complex.

We investigated the structural heterogeneity of the 19S regulatory complex by crosslinking experiments (Kurucz et al. 2002). Some of our antibodies, that recognise the same subunit, give different crosslinking pattern, suggesting that the regulatory complex may contain these subunits in two different steric conformations. Such a conformational heterogeneity may be the consequence of posttranslational modification.Therefore we investigated the posttranslational modifications of the 26S proteosome subunits with wheat germ agglutinin (WGA) and monoclonal antibodies specific for N-acetylglucosamine modification(MA-072,MA-076). We managed to show that some subunits of the 20S core particulum and the 19S regulatory complex are O-glycosilated.

For fuctional experiments we are setting up an in vitro ubiquitination-protein degradation assay. We want to combine chromatographically purified enzymes (E1) with recombinant proteins (E2, Vihar), and imunoprecipitated enyimes (E3,APC) preactivated with recombinant protein factors (Fizzy, Fizzzrelated). Using chromatographycally purified active 26S proteosome we would like to investigate in detail the multiubiquitination and the degradation steps of the ubiqutin-proteasome pathway. We have already produced most of the componets of this system, and now we are working on the activation of the E3 APC component.

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DISSERTATION SUMMARY

Genetic polymorphism in Cumanian population determined by analysis of ancient bone samples

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In the Hungarian village of Csengele, on the borders of what is still called Kiskunsag, an archaeological excavation in 1975 revealed the ruins of a mediaeval church with 38 burials. Supported by historical data archaeologists have established that the buried persons belonged to the Cumanian ethnic group. In 1999, the grave of a high-status Cumanian was discovered, about 50 meters from the church of Csengele, which could prove the ethnic identity of the former excavated remains.

The ethnic origins of the Cumanians are uncertain, although their anthropological characteristics suggest that their geographical origin might be in Inner-Asia, South-Siberia. According to historical data after the ravage of Hungary by Tartar hordes, the Cumanians settled down on the partially uninhabited area of the early Hungary. So far these ethnic groups both genetically and culturally completely assimilated into Hungarians. In order to study the genetic origin and relationships of an ethnic group in such cases, analyses of ancient DNA (aDNA), obtained from archaeological bones of very early settlers of that particular ethnic group, could be the only reliable solution.

Mitochondrial DNA (mtDNA) is commonly used in aDNA studies because of its high copy number and the lack of recombination due to its exclusively maternal inheritance (Giles et al. 1980). The hypervariable region 1 (HVR-1) of the mitochondrial control region is routinely used in human phylogeographic studies, because it evolves 10 times faster than the protein coding-region of mtDNA (Richards and Macaulay 2001; Vigilant et al. 1991). Human mtDNA HVR-1 mutations have accumulated sequentially along radiating maternal lineages, during and after the process of human colonization of different geographical regions of the world (Marjoram and Donnelly 1994). Hence, haplogroups (groups of mitochondrial DNA types) often show geographic specificity (Torroni and Wallace 1995). Analysis of mtDNA in populations therefore allows reconstruction of their maternal lineages, makes it possible to study the genetic traces of migration and admixture of different human communities, and helps to estimate the degree of relationships within and between populations.

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To determine the genetic background of ancient Cumanian population, DNA was extracted from 11 bone samples. The nucleotide sequences of the mitochondrial HVR-1 were determined. To define the haplotypes and the haplogroups, where the Cumanian samples belong to, the HVR-1 polymorphic sites and in problematic cases the haplogroup–associated SNPs in the mitochondrial protein coding-region were determined. A database was set up from previously published mtDNA HVR-1 sequences, representing 7,099 persons from 153 different worldwide populations. Median-joining networks were created to assign the relationships between Cumanians and the components of our database. The maternal origin of the Cumanian samples examined was determined through these phylogenetic networks.

A genetic method based on X and Y chromosome specific alphoid satellite markers (Lin et al. 1995) was applied to determine the gender of the bone material from an infant and a juvenile individual and for confirming the anthropological sex identification of four adult individuals.

It was established that the Cumanian population derived from the excavation was not genetically homogenous. According to our results the Cumanians could be originated from Central-East Asia. As a result of genetic admixture those Cumanians, who settled down 800-900 years ago in Hungary, contained several European genetic elements.

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Free Radical Research – 2002

Symposium

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Csongrád County Group of the Association of Hungarian Chemists
University of Szeged

Held in the House of Technology

in Szeged, Hungary, on September 27-28, 2002

Invited Lectures

Guest Editor: Ilona Szöllősi Varga

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SYMPOSIUM

In memory of Professor Béla Matkovics on the occasion of the 75th anniversary of his birth

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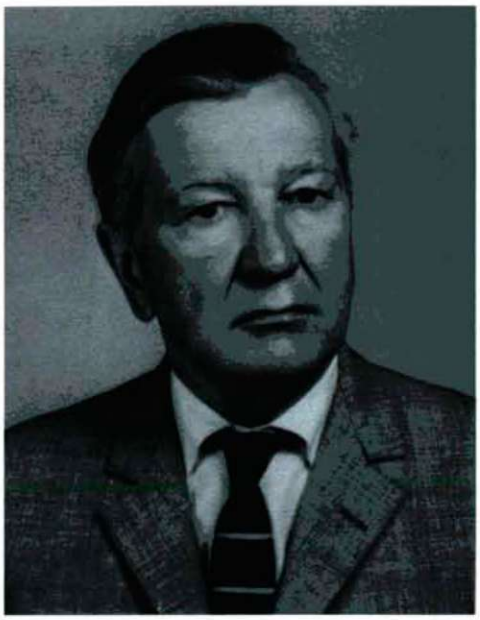

On September 27-28, 2002, the Hungarian Free Radical Research Society, the Csongrád County Group of the Association of Hungarian Chemists, and the University of Szeged held a joint scientific meeting in the House of Technology in Szeged. With the scientific programme entitled "Free Radical Research 2002" we paid tribute to the memory of Professor Béla Matkovics on the occasion of the 75th anniversary of his birth.

Béla Matkovics was born on 12 July, 1927 in the Hungarian town Csongrád. Following schooling in Bonyhád and Baja, he enrolled as a university medical student in the city of Szeged, and received his medical diploma in 1951. He then joined the staff of the Department of Medical Chemistry of the Medical University in Szeged. Additionally, however, he continued his studies, and in 1955 he graduated in chemistry from the University of Szeged. From this time on, he served on the staff of the Department of Organic Chemistry of that university. Among his more memorable achievements were the organisation and commencement of teaching in biochemistry within the programme for the training of biologists, wich began in 1964. He founded and directed the Biochemistry and Genetics Group at the University of Szeged, which subsequently became the Biological Isotope Laboratory.

In 1964, Béla Matkovics received the title of Candidate of Chemical Science from the Hungarian Academy of Sciences, and in the same year he was awarded his university doctorate. In 1994, he was granted a Doctorate in Biological Sciences by the Hungarian Academy of Sciences. In 1995, he was appointed a full university professor. He retired in 1997.

His scientific activities initially involved the microbiological transformation of organic compounds. He then turned to the investigation of steroids, redox systems, oxidative transformations, and pathological changes caused by active oxygen radicals.

Besides his scientific and teaching work, he found time to play an active role in the Csongrád County Group of the Association of Hungarian Chemists. He participated untiringly in the organisation of the series "Protein Chemistry Practicals" and "Szeged Course in Chromatography". Unfortunately, the 30th of the latter course, in January 1999, had to be held without him.

Rather than listing of his scientific achievements, his publications and his awards, stress should be laid here on the "paternal" care and selfless readiness to help which he constantly demonstrated towards his colleagues and students during so many years. The opportunity to learn to master a host of fundamental methods was ensured for a whole series of young researchers, all of whom were soon burrowing deeply into the literature. Independently of what topic he happened to be working on, he shared his research fields with many colleagues.

Professor Matkovics created a school in the field of research into free radicals in Hungary, with particular regard to oxidative stress and antioxidants. Virtually all of those currently carrying out research in this field in Hungary began on the basis of his instructions and guidance. The present writer considers herself highly privileged to have had the opportunity to have been among his students, and to have been his colleague for close to 30 years.

Sadly, at the height of his scientific powers,he was taken away from us extremely suddenly on October 13, 1998. However, as clearly revealed at the meeting held in his honour, his memory still lives on with tremendous and wide-ranging affection and respect.

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SYMPOSIUM

An overview of free radical research*

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ABSTRACT The discovery of the pathogenetic role of free radicals in various human and animal diseases initiated a wide range of investigations, resulting in a number of important scientific dicoveries. These are reviewed in this paper, with an emphasis on preventing atherosclerotic complications and plaque development. The administration of antioxidants in the prevention and therapy of different diseases is listed, too. The questions of optimum antioxidant supply to prevent atherosclerosis, malignancies and other diseases are discussed.
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KEY WORDS

free radical
antioxidant
reactive oxidative substance
atherosclerosis

The discovery of free radicals (FRs) and their pathogenetic role in the development of atherosclerosis (AS), tumours, teratogenesis and ageing initiated a wide-ranging research on this field (Horváth and Jávor 1985; Fehér et al. 1993; Frei 1994; Bergendy et al. 1999). This area includes the ecology and environmental medicine and has implications in the biological, biochemical or pharmacological investigations, as well as in the agriculture and in the veterinary and human medicine.

Medical and public health implications

In the "civilised" life the quantity of FRs has grown enormously. Oil the main source of energy in the world, the smoky atmosphere contains a lot of FRs generated by the burning process (Gracy et al. 1999; Hiura et al. 1999; Velichovszkij 2001). Xenobiotics are widely dispersed, as well. The organochlorides have become ubiquitous. These compounds are not only neurotoxic, but they also increase the superoxide production in the mitochondria and cause oxidative damages to the DNS (Stedeford et al. 2001).

These toxic substances might be concentrated in some foods. A sad consequence of this harmful accumulation is the diminished fertility of eskimoes brought about by the xenobiotics found in their principal nutrient, in the liver and oil of narwals.

It is an intriguing question as to how long time can nature compensate for this flood of FRs and which diseases or mutations could arise.

The mass of FRs affecting the mankind has been multiplied in the last 100 years, while, in contrast, the antioxidant (AO) intake of people has diminished. The consumption of fresh fruits and vegetables is very low, whereas smoking, alcohol, obesity, accompanied by a lot of faulty eating and culinary habits increase the FR load to the human organism (Fehér et al. 1993; Tulok and Matkovics A 1997).

The role of antioxidants in maintaining health

At the beginning of FR research a much expected possibility was to prevent the AS and tumour development by neutralizing FR pathogenicity (Horváth and Jávor 1985). A tremendous amount of scientific investigations was carried out. More and more AO substances have become known to provide the biomolecules an effective AO protection. Various kinds of fruits, vegetables and medicinal plants were examined and their abundance in AO came to light (Fehér et al. 1993).

The AOs differ in their affinity to the various FRs as well as in their binding site in the organism, *e.g.* vitamin E is highly effective with the superoxide radicals but it is very weak in scavenging hydroxyl ions. The "master of AOs" is vitamin E, but on the surface of biological membranes and of the blood lipids only, whereas in the fluid spaces vitamin C is the main AO, as glutathion acts intracellularly and melatonin in the nerve cells. Thus: the AOs can be classified according to their FR affinity and tissue concentrations (such as antibiotics can). It is beyond doubt that there is neither a generally effective AO compound, nor a universal AO treatment (Matkovics A 2001). There are only some substances scavenging certain FRs on defined cells or molecules.

When determining the AO content of a tissue or plant the result depends on the method employed (FRAP, TAS, TBARS, etc.). Testing different fruits in a peroxide system plums, bananas and red grapes were the most effective against FRs, whereas in a hypochlorite system the banana proved to be the weakest. Similarly, examining some food additives (butylhydroxytoluene, trinatriumpolyphosphate, phenol, propylgallate, etc.) and spices (rosemary, red pepper and oregano as the best ones) a different rank order was

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established in the two mentioned systems, except vitamin E, which proved to be the weakest one (Murcia et al. 2001). Garlic, carrots, apricots, onions, curry, citrus fruits and kale sorts had good AO properties as well.

The advantages of tea and fish consumption are undoubtedly proven, but the role of red wine in preventing AS is still debated. The publication of the "french paradox" prompted an intensive investigation. The results are controversial, but they suggest slightly, that a moderate drinking of red wine (1- 3 dl daily but not more!) may be protective against AS (Goldberg et al. 2001), and, possibly, against neoplastic diseases too. The differences of the studies on this subject may be explained by the individual polymorphism of alcohol metabolizing enzymes and by the different populations among which these investigations were performed.

Adverse effects of antioxidant administration

A great number of data shows that any AO can be transformed to a prooxidant, especially if it is given in a high dose and without other AOs. When being not able to step in to the chain of electron transport the AO will be saturated and become a FR source. Probably this is the explanation of the failure of the „Natural" Cancer Prevention Trial (Peterson 1996). Later it was experimentally proved that high doses of ß-carotene produce superoxide radicals.

It can, thus, be stated: "A single antioxidant is not an antioxidant!" (Tulok and Matkovics 1997).

A new term called "antioxidative stress" has also been introduced. This includes all adverse effects of AOs and the indirect disadvantages of them. For example, the beneficial action of exercise by the induction of aortic catalase activity and endothelial NO synthase expression was counteracted by the administration of vitamin E (Meilhac et al. 2001). It means that an adaptative reaction may be hindered by the administration of an AO. Certainly the physiological FR reactions must not be blocked. The AOs have a Janus face!

Prevention of diseases by antioxidants

The best results of AO administration were attained in halting the progression of AS.

The first interventional trials with the administration of vitamin E did not yield convincing results. Although vitamin E in chronic renal insufficiency reduced the number of cardiovascular events by 40%, it is known, that in this condition the atherosclerotic process quickens by up to 6 – 26% faster, than in the age-matched control people (Boaz et al. 2000). Other studies gave minimal or negative results. These latter ones, however,were performed on people who probably had had AS plaques already at the beginning of the study.

Hence it can be suggested that vitamin E really hinders only the initial phase of AS, but has not any important influence on the later processes of plaque development and rupture, leading to myocardial infarction or arterial occlusion, to the so- called "hard endpoints."

Although the early captopril studies (SAVE, SOLVD) showed a significant reduction in stroke and cardiovasular events, the HOPE study (The Heart Outcomes Prevention Evaluation Study Investigators, 2000) provides the convincing evidence for the effectivity of ACE inhibitors in the impeding the AS process in the vascular wall.

Contemporary laboratory investigations have pointed out the pathogenetic role of angiotensin-II (which is also a ROS producer) in the process of AS: oxidizing lipoproteins, activating macrophages and smooth muscle cells, triggering the excretion of cytokines, adhesion – and apoptotic factors, leading to plaque formation – thus verifying theoretically the beneficial effects observed by the ACEi administration.

The administration of calcium channel antagonists and the angiotensin AT_1 blockers in hypertensive patients and HMG-CoA reductases in hyperlipidemic patients provided similar positive results to the aforementioned ones.

Corti and his co-workers measured the size of AS plaques with a high resolution MRI method and found out that after a year of simvastatin therapy both the diameter of AS plaques and the thickening of the arterial wall were signif-icantly diminished (Corti et al. 2001).

Such regression of AS plaques (in 15-18% of treated patients) has been already observed in some studies on antihyperlipidemic treatment both in the ELSA study with lacipidin and in other statin and fibrate studies.

The atherogenetic role of hyperhomoscystinemia was discovered in the latest years, with the hope to prevent AS in these cases with the administration of folic acid and vitamin B_6.

The main causes of AS are summarized in Table 1.

In the prevention of neoplastic diseases no similar achievements could have been reached. A number of studies on successful prevention were published (Shanghai, New Zealand, Uruguay study, etc.) but it is not clear, what kinds of materials and in which combination and doses are necessary.

The key question of cancer prevention and avoidance of premature ageing seems to be the AO protection of the DNS and the mitochondria (Van Remmer and Richardson 2001; László and Falus 2002). An immense research is going on for newer and better mitochondrial and DNS-protecting AO substances.

Antioxidants in the therapy

Every illness or tissue damage leads to an increased FR generation and these radicals cause further injuries (Matkovics 1993). Therefore each disease leads to a common, unspecific complication: the oxidative stress. So: the administration of

Table 1. Factors of atherogenesis.

Cause	Pathomechanism
Hypertension	Endothel dysfunction and lesion Biomechanical (shear) stress Overproduction of endothelin and angiotensin
Hyperlipidemias	Oxidised LDL Lipoprotein-A ; remnant- lipoproteins Triglyceride and free fatty acids Leptin Deficiency in HDL
Hypercholesterinemia Dyslipidemia Obesity Metabolic X syndrome	
Hyperfibrinogenemia Hypercoagulation syndromes	Enhanced activity of thrombocyte mitogenes and LDL binding capacity of the vascular wall ncreased smooth cell proliferation and migration.
Smoking	Increased lipid peroxidation Oxidative stress in the vascular wall Vasoconstriction, ischaemia , generation of ROS
Hyperhomocysteinemia	
a) congenital, (b) deficiency in vitamine B_6 and c) folic acid d.) Diabetes mellitus type 2. (NIDDM)	Autooxidation products of ROS (peroxide and superoxide radicals) Inhibition of glutathion peroxidase
Generalized oxidative stress Critically severe conditions:burns, polytraumatization, respiratory distress syndrome, serious infection, etc.)	Increased lipid peroxidation Endothelial damage caused by ROS
Nutritional AO deficiencies, alcoholism, Abundant lipid intake (including PUFA)	Weakness in defense to ROS Oxidative stress Increased lipid peroxidation
Ageing	Increased production of mitochondrial ROS. Oxidation of membrane lipids Lipid peroxidation enhanced.
Diabetes mellitus (mainly Type 2)	Glycation products, glycotoxicity, lipotoxicity
Chronic renal insufficiency	A general increase in ROS production Enhanced lipid peroxidation
Infections, inflammations, autoimmune diseases (SLE) (Chlamydia, CMV, herpes viruses, oral pathogenes, focal infections, etc.)	Increased inflammatory reactions in the vascular wall

AOs is reasonable in all diseases, in the form of the poly-AO therapy. The results of this approach have already been published in a lot of articles. In cases of polytraumatized patients (Porter et al. 1999) burns and in other critically serious conditions a higher percentage of recovery was observed during the AO treatment (N-acetylcysteine, selenium, Vitamin C+E, allopurinol and lazaroid combination): with a smaller rate of infections, cardiovascular or other complications (Bulger and Maier 2001). In subarachnoidal hemorrhages a smaller percent of brain damages was found in the AO group than in the control ones (Asano and Matsui 1999). Similarly, Goode (1993) observed hopeful outcomes in sepsis cases by the combined AO administration.

Professor Boda had been investigating the uric acid metabolism since the 60's and based on his own results he saved the life of many children with serious conditions (such as shock, or respiratory distress syndrome) by the allopurinol treatment (Boda and Németh 1983). It came to light only later, that in such clinical states the xanthine oxidase activity has increased, resulting in a high overproduction of superoxide radicals.

In the course of the influenza the ROS derived from the macrophages causes more damages than the influenza virus itself (Jacoby 1994). Hemila and Douglas (1999) pointed out, that vitamin C is really effective in acute respiratory infections, but it must be given in high doses, such as 2000 mg daily.

The combinations of AO compounds (mostly based on silymarin with other AOs) has proved to be effective in the therapy of different (toxic and infective) hepatic diseases (Berkson 1990; Fehér and Vereckei 1990; Fehér 2002). Good effect was observed with a vitamin C, coenzyme Q_{10}, ß-carotene combination in primary biliary cirrhosis (Watson et

al. 1999). The chronic pancreatitis was influenced favourably with selenium compounds (Bowrey et al. 1992).

A vast body of literature testifies that a row of renal (Annuk et al. 2001; Clermont et al. 2001), hematological (Németh et al. 2000), autoimmune, gynaecologic, neurologic and metabolic disorders have been influenced favourably by the AO treatment, as summarized by Matkovics (2001).

There is a large quantity of materials which are effective against experimental carcinogenesis or they cause apoptosis of tumour cells – but not in curing human malignancies. In the malignant cell lines the pycnogenol, allicin and resveratrol showed the greatest antitumour activity. The clinical results have been scarce until now. Lockwood et al. (1994) published surprisingly good effects with a megadose coenzyme Q_{10} and multivitamin therapy, but these are still not confirmed by other investigators.

In clinical oncology the AOs have at present a rather adjuvant role, *e.g.* glutathion alleviates the toxic side effects of cysplatin therapy.

Some open questions

According to our present knowledge, the most important questions are still to be answered, *e.g.*: What does the optimal AO supply consist of? How many AOs must be taken and in what dosage? How can we provide a safe AO protection for the most important biomolecules without any harmful effect?

Nowadays the wisest advice might be: taking as many kinds of natural AO-s as it is possible, and in proportions resembling natural foods.

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SYMPOSIUM

Reducing power of the natural polyphenols of *Sempervivum tectorum* in *vitro* and *in vivo*+

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ABSTRACT Unhealthy dietary habit *e.g.* lipid rich diet could result in inflammatory processes and necrosis in the gastrointestinal tract in which free radical reactions are involved. Higher plants have many free radical scavenger molecules and anti-inflammatory compounds in wonderful variations. *Sempervivum tectorum L.* is well known plant in folk medicine. In the case of complex plant extracts, such as *Sempervivum tectorum* ones, which contain several active compounds, it is impossible to discover the single mechanism of action, therefore practically the reducing activity have to be measured *in vitro* and in rat intestinal tract *in vivo*. The question was, whether the absorbed active compounds of *Sempervivum tectorum* extract were able to influence on the changed cellular redox states in mucosa of all part of intestinal tract in experimental bowel disease. This biochemical work presents the protective effect of natural polyphenols and flavonoids of *Sempervivum tectorum* extract in bowel disease - especially in jejunum and ileum - induced by high dietary triglyceride and cholesterol level in rats.

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KEY WORDS

Sempervivum tectorum
redox-homeostasis

It is generally agreed that the composition of diet plays a fundamental role in the induction of intestinal cancers. The redox homeostasis is very important for the equilibrium among cancer development, apoptosis, regeneration and necrosis. The moderate nutritional habits with natural antioxidants can help to restore the normal function of gastrointestinal tract, but the inadequate consumption of vitamins and polyphenol type antioxidants is contraindicated (Jadoul et al. 1993; Lugasi et al. 2000; Blázovics et al. 2002).

Cells and tissues have specific defence mechanism against pathological free radical processes. Certain activities or concentrations of antioxidants themselves (enzymes, vitamins or function groups) do not represent the antioxidant states of specimens (McCord 2000; Blázovics et al. 2002). Therefore a non-specific biochemical measurement, the reducing power was applied for the detection of antioxidant states of bowel parts to evaluate the redox states of mucosa homogenates in rats (Oyaizu 1986).

The judgement of biological and pharmacological effects of quercetin and kaempferol is extreme in literature, similarly to the polyphenolic compounds; their mutagenicity and carcinogenicity are supposed in biological relevant pH. In spite of that these two antioxidant flavonoids are ubiquitous in almost every foods of plant origin (Hertog et al. 1992; Bors et al. 1996; Hertog and Hollman 1998; Lugasi 2000).

Sempervivum tectorum extract contains approximately 20 different flavone and flavonol mono-and diglycosides (0.7 w/w%), mainly quercetin and kaempferol glycosides, polyphenolic compounds (4.2 w/w%), *e.g.* proanthocyanides, phenol carboxylic acids, ascobic acid, 11.2 w/w% polysaccharides and micronutrients, mainly Ca (76.52 mg/g), K (40.47 mg/g), Mg (17.85 mg/g). Characteristic monosaccharides are rhamnose, arabinose, xylose, mannose, galactose and uronic acids after strong hydrolysis. Alkaloids could not be detected in any examined samples (Blázovics et al. 1992 a,b,c, 1994; Abram and Donko 1999).

Data for toxicity after i.p. administration are: in male rats LD50 value is 2276 mg/bw kg and in female rats LD50 value is 2098 mg/bw kg, maximal tolerance i.p. 500 mg/bw kg and p.o. 5000 mg/bw kg in both sexes. These data indicate that the administration of *Sempervivum tectorum* extract represents a very low risk (Blázovics et al. 2002).

In previous studies we established that *Sempervivum tectorum* extract has not toxic effect at all on whole body in spite of its high quercetin and kaempferol contents in the applied concentration (2g/bw kg/day for 10 days) and we gave account of antioxidant, free radical scavenger (by EPR technique), membrane protecting, immune stimulating, serum lipid level lowering and HDL-cholesterol enhancing properties of *Sempervivum tectorum* extract *in vitro* and *in vivo* studies in concordance with literature.

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+In memory of Professor Béla Matkovics

Materials and Methods

The lyophilised extract of *Sempervivum tectorum* L. (Crassulaceae) was used for all examinations as a standardised preparation by 207657/1993 SOTE Patent. Serum bovine albumin was from Calbiochem AG (Lucerne). All other reagents were purchased from Reanal (Budapest).

Animal experiments

Forty Wistar rats (150-200 g) (obtained from Charles River Hungary Kft.) were divided into four groups (N=10-10) (control and fat rich diet fed and treated with extract in two adequate groups). Lipid rich diet (2% cholesterol, 0.5% cholic acid, 20 % sunflower oil added to the rat chow) was applied for the experiments. Extract was dissolved (2 g/bw kg) in the daily drinking water and added parallel with feedings for 10 days. At the end of treatment the animals were exsanguinated from abdominal vein in deep pentobarbital narcosis (55 mg/bw kg; Blázovics et al. 1992a).

Reducing power property

The reducing power of the samples was determined according to the method of Oyaizu (1986) based on the chemical reaction of Fe(III) => Fe(II). The absorbance of the reaction mixture was read at 700 nm. Increased absorbance indicated increased reducing power.

Preparation of bowel mucosa

After identification of bowel parts (duodenum, jejunum, ileum, coecum, colon, rectum), the bowel was cut and the content of actual bowel part was eliminated tenderly and washed three times with isotonic ice-cold NaCl solution. Mucosa was harvested by tender power with blunt knife using microscopic control.

Protein content

Protein content of mucosal homogenates was measured by Lowry et al (1951). Protein concentration of all bowel part mucosal homogenates was adjusted 10 mg/ml using bovine albumin as standard for the measurements.

Statistical analysis

Each value represents the mean ± SD of different measurements. In *in vitro* studies 5 parallel measurements were carried out, and in the animal experiments 2-2 parallel were in each measuring point in each animal. Significance was established at $p<0.05$ probability level.

Results

Reducing power was detected *in vitro* as a control for the *in vivo* experiments. Table 1. shows the reducing power of *Sempervivum tectorum* extract *in vitro* applied ascorbic acid as a standard. Reducing power was dependent on concentration.

Figure 1. shows the basic reducing power of different bowel mucosa. Significant difference can be seen in reducing capacity between small and large parts of intestinal tract. Injurious effect of fat rich diet changes the reducing power in all parts of intestinal tract (Figure 2). The significant difference is remained between small and large bowel, as well. Reducing power of duodenum, jejunum, ileum was significantly higher than in coecum, colon, and rectum.

The redox balance was not changed significantly, when the normolipidemic animals were treated with this extract. Results are not shown.

Antioxidant treatment caused a significant beneficial change in reducing power of ileum homogenate and non significant in coecum, colon and rectum as can be seen in Figure 3.

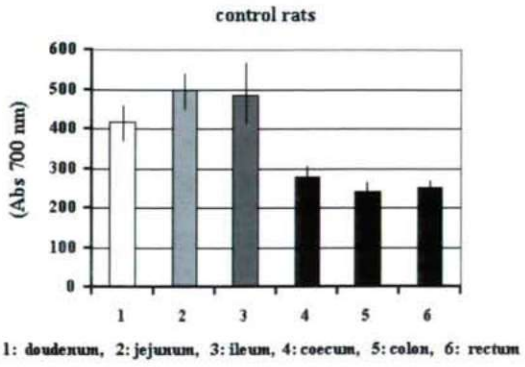

Figure 1. Reducing power in homogenates of small and large bowel mucosa in normolipidemic rats.

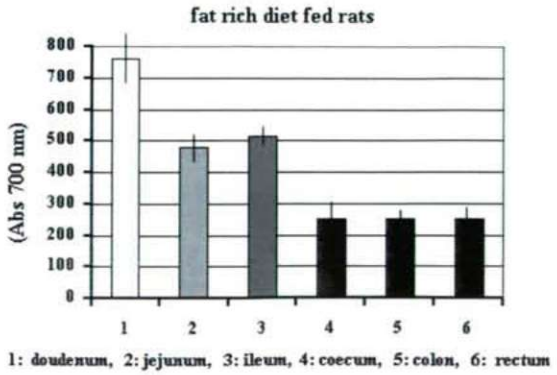

Figure 2. Reducing power in homogenates of small and large bowel mucosa in hyperlipidemic rats.

Table 1. Reducing power of ascorbic acid and *Sempervivum tectorum* extract *in vitro*.

Ascorbic acid Concentration (mg/ml)	Absorbance (700nm)	Extract Concentration (mg/ml)	Absorbance (700nm)
0.01	0.067 ±0.067	0.25	0.211 ±0.004
0.02	0.123 ±0.005	0.50	0.321 ±0.004
0.05	0.316 ±0.003	1.25	0.659 ±0.010
0.10	0.584 ±0.009	2.50	0.668 ±0.004

Discussion

Antioxidant, superoxide ($O^{\bullet}_2$) and hydroxyl radical ($^{\bullet}OH$) scavenger properties of *Sempervivum tectorum* extract were proved by EPR spectroscopic and chemiluminometric techniques (Blázovics et al. 1992c). Potential bioactive constituents were determined by chromatography (HPLC, TLC) and spectrometric (UV, UV-VIS) methods. It was established that activities of NAD(P)H reductases and content of cytochrome P450 were normalised in hyperlipidemic rat liver microsomes, if the animals were treated with this extract. Fatty acid composition in the liver was changed beneficially examined by HRGLC analysis. NADPH induced lipid peroxidation was also decreased in microsomes in *in vivo* and *in vitro* experiments. In the same time the components of *Sempervivum tectorum* had no significant influence on MFO system in normolipidemic animals and on cytochrome b5 concentration of microsome fractions of hyperlipidemic rats (Blázovics et al. 2000, 2002). Favourable changes of ion concentration were observed in the bile fluid of hyperlipidemic rats treated with extract (Szentmihályi et al. 1999, 2000).

Histological studies supported that enormous changes happened both in the liver and in jejunal mucosa of animals kept on fat rich diet. *Sempervivum tectorum* extract could restore the tissue structures (Blázovics et al. 1992, Fehér et al. 1992).

In the present experiments we justified the concentration dependent reducing ability of *Sempervivum tectorum* extract *in vitro* comparing with ascorbic acid and its beneficial effects on reducing property of bowel mucosa in animal experiments. The measurement of reducing power is suitable for study the complex effect of natural extracts as well as investigate the redox homeostasis of tissue homogenates.

The reducing power was very high in duodenum of animals kept on fat rich diet, but the data were not changed significantly in jejunum, ileum, coecum, colon and rectum. The investigation of the reason of these changes needs further efforts.

Antioxidant compounds of *Sempervivum tectorum* extract caused significant elevation of reducing power of ileum mucosa in hyperlipidemic rats, and smaller reducing activity in parts of large bowel.

The relatively high essential metal ion content of extract (Blázovics et al. 2002) probably contribute to the favourable effect on bowel mucose. Especially zinc and magnesium may have a significant role. Zinc as an antioxidant element directly and magnesium by formation MgATP indirectly may participate in the operation of antioxidant defense system (Lakatos et al. 1997).

It can be known from the literature, that the fenolic acids, sourced from flavonoids can absorb in large bowel. Therefore, we concluded from the results that antioxidant compounds in basic form and after bacterial enzymatic transfor-mation in large bowel take part in redox homeostasis of intestinal tissues.

On the bases of our experimental data we proved that the antioxidant power of this drug extract was manifested in the bowel parts as well as in the liver.

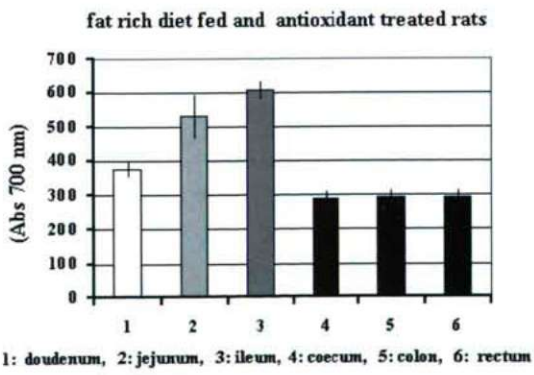

Figure 3. Reducing power in homogenates of small and large bowel mucosa in hyperlipidemic rats treated wit *Sempervivum tectorum* extract.

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SYMPOSIUM

An evaluation of the antioxidant abilities of *Allium* species+

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ABSTRACT In the present study we investigated antioxidative properties of leaves of different wild (*Allium flavum* L., *Allium sphaerocephalum* L., *Allium atroviolaceum* Bois., *Allium vienale* L., *Allium scorodoprasum* L.) and grown (*Allium nutans* L., *Allium fistulosum* L., *Allium vienale* L., *Allium pskemense* B. Fedtsch, *Allium schenoprasum* L., *Allium cepa* L., *Allium sativum* L.) *Allium* sorts were investigated. Activities of antioxidant enzymes (superoxide dismutase, catalase, peroxidase, glutathione peroxidase), quantities of malonyldialdehyde superoxide and hydroxyl radicals and reduced glutathione and also the content of total flavonoids, chlorophylls a and b, carotenoids, vitamin C and soluble proteins were determined. Our results indicate that leaves of grown *Allium sativum* L., *Allium cepa* L., *Allium vineale* L., *Allium fistulosum* L. and *Allium nutans* L. and wild *Allium flavum* L. and *Allium ursinum* L. exibited high antioxidant activites.

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KEY WORDS

antioxidant enzymes
flavonoids
chlorophyll
carotenoid
Allium species

Oxygen free radicals – highly reactive species ($O^{\bullet}_2^-$, $^{\bullet}OH$, $HO^{\bullet}_2$) with one or more unapaired electrones, H_2O_2 and activated oxygen species ($^1\Delta g$, $^1\Sigma g$) are formed in almost every cell of the body at an astonishing rate during normal oxdative metabolism (Gey 1994). Environmental factors such as UV light, ozone, tobaco smoke, different xenobiotics, herbicides, pesticides, ionizing radiation etc. cause their formation in greater extent (Halliwel and Gutteridge 1984). They react voraciosly with almost every cellular component and contribute to many types of pathology. Antioxidant defense mechanism counteract free radicals formation and reactions. In free radicals caused pathology antioxidants neutralize free radicals and increasing levels of antioxidants should decrease pathology. Combinations of different natural antioxidants which could be find in different medicinal plants work better that separate antioxidants alone. Many epidemiological studies also support the idea that antioxidants are interdependent (Kery et al. 2001).

Onions are widely used in all parts of the world as a flavoring vegetable in various types of food. According to traditional medical literature they are source of many vitamins and are useful in fever, dropsy, catarrh and chronic bronchitis (Block 1985). Roasted or otherwise they are applied as a poultice to indolent boils, bruises, wounds, to relieve hot sensations and applied to the navel for dysentery and fever (Brewster and Rabinowitch 1990).

Today *Alliums* are used for their flavor, aroma and taste, beeing prepared domestically or forming raw material for a variety of food manufacturing processes (dehydration, freezing, canning and pickling). Also, dehydrated onion production is widely used, especially in the manufacture of other processed foods (Brewster and Rabinowitch 1990). On the other hand therapeutic and medicinal values of garlic and onions are the subjects of many researches. The different clinical studies have shown their benefit in the reduction of cardiovascular disease risk by inducing lowering of serum cholesterol and blood pressure (Steiner and Lin 1994). They have liver protective (Dion and Miler 1996), imune enhancement and anti-infection (Lau 1989), anti-stress and anti-fatigue (Kawashima 1986), anti-cancer and cancer preventive effects (Dion and Milner 1997; Pinto et al. 1997; Balasenthil et al. 2001), brain and neurotrophic (Moriguchi 1996) and other pharmacological effects (Yeh 1996). Many recent studies showed that *Alliums* have antioxidant effect what could be of the great importance for its use in prevention and treatment of different diseases (Lau 1989; Numagami 1996; Geng and Lau 1997) and contribute to its therapeutic physionomy (Kyo et al. 1998).

Our previous studies (Stajner et al. 1998a; 1988b; 1998c, 1999) showed that different *Allium* species posses well-defined antioxidant activity. Therefore the aim of this study was to perform the screening of different *Allium* species by determination activities of antioxidant enzymes superoxide dismutase (SOD), catalase (C-ase), peroxidase (P-ase), glutathione peroxidase (GP-ase), quantities of malonyldialdehide (MDA), superoxide ($O^{\bullet}_2^-$) and hydroxyl radicals ($^{\bullet}OH$), reduced glutathione (GSH) and contents of total flavonoids, chlorophylls a and b, carotenoids, vitamin C and soluble proteins. Our results could evaluate their antioxidant values and point to easy accessible sources of natural antioxi-

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+In memory of Professsor Béla Matkovics

Table 1. Enzyme activities in leaves of different *Alliums*.

Allium sort U/mg protein	SOD	C-ase	P-ase	GP-ase
Allium sativum L.	52.47±7.11	8.78±0.43	40.48±4,01	0.038±0.011
Allium vineale L. (grown)	39.95±2.34	23.77±15.92	8.54±0.40	0.035±0,010
Allium vinaele L. (wild)	3.63±0.52	7.32±4.54	22.51±2.26	0.020±0.006
Allium sphearocephalum L.	8.91±2.52	6.42±1.10	105.83±8.00	0.050±0.014
Allium scorodoprasum L.	45.54±23.88	7.32±11.96	130.10±10.00	0.977±0.052
Allium atroviolaceum Boiss.	7.46±1.51	8.81±6.63	17.06±1.18	0.048±0.004
Allium cepa L.	2.69±0.76	5.51±3.13	10.23±0.91	0.051±0,011
Allium pskemenese L.	4.08±0.84	3.16±1.25	4.87±0.04	0.065±0,049
Allium fistulosum L.	62.20±3.85	20.59±11.89	23.28±0.88	0.537±0.043
Allium schenoprasum L.	77.46±5.98	5.91±0.62	63.26±4.21	0.083±0.024
Allium nutans L.	20.27±3.27	10.01±5.11	10.46±0.45	0.018±0.002
Allium ursinum L.	2.51±1.21	11.48±6.90	8.85±0.19	0.052±0.007
Allium flavum L.	10.62±2.02	13.59±8.28	21.03±1.17	0.022±0.005

dants that could be used as a possible food supplements or in the cosmetic and pharmaceutical industries.

Materials and Methods

The leaves of next wild (*Allium flavum* L., *Allium sphaerocephalum* L., *Allium atroviolaceum* Bois., *Allium vienale* L., *Allium scorodoprasum* L.) and grown (*Allium nutans* L., *Allium fistulosum* L., *Allium vienale* L., *Allium pskemense* B.Fedtsch, *Allium schenoprasum* L., *Allium cepa* L., *Allium sativum* L.) *Allium* sorts were investigated.

One g of plant material was ground with quartz sand in a cold mortar. The ground material was suspended in 5 ml 1 mol/l K_2HPO_4 at pH 7.0. After a 10 min centrifugation at 4°C and 15,000 g, the aliquots of the supernatant were used for SOD activity measurements. 20 µl of Tsuchihashi solution (chloroform:ethanol – 3:5) was added to the supernatant prior to measurement of the enzyme activity. The SOD activity was determined in aliquots by the method of Misra and Fridovich (1972) based on the inhibition of transformation of adrenaline to adrenochrome at pH 10.2 (Matkovics et al. 1977).

For the other antioxidant enzymes and biochemical determinations, the plant material was treated in the same way but the medium was 0.1 mol/l phospthate buffer (pH 7) with a plant material to medium ratio of 1:5, centrifuged for 10 min at 15.000 g. After the centrifugation the supernatant was evaluated for: P-ase activity, using guiacol as substrate (Matkovics et al. 1977); GP-ase activity using cumene hydroperoxide and GSH as substrates (Chiu et al. 1976); C-ase activity spectrophotometrically at 240 nm (Simon et al. 1974); lipid peroxidation by the thiobarbituric acid (TBA) method; values were given as equivalent amounts od MDA; the calibration curve was prepared with malonyldialdehyde bis-diacetal (Placer et al. 1968); superoxide radical was determined by adrenaline autooxidation (Misra and Fridovich 1972); hydroxyl radical by the inhibition of deoxyribose degradation (Cheesman et al. 1988).

The amount of GSH was determined with Ellman (Sedlak and Lindsay 1968) and protein by Folin reagents (Lowry et al. 1951).Total flavonoids were estimated according to Marckam (1989). Pigments were extracted with acetone and determined spectrophotometrically using molar extinction coefficients according to Wettstein (1957). The content of vitamin C was determined according to AOAC Official Methods of Analysis (1984).

Table 2. Quantities of ($O^{\bullet}_2^-$), ·OH and MDA in leaves of different *Alliums*.

Allium sort	($O^{\bullet}_2^-$)	·OH (nmol/mg protein)	MDA
Allium sativum L.	508.8±218.1	2.10±0.29	24.55±1-02
Allium vineale L. (grown)	155.9±78.0	2.32±0.05	13.19±0.51
Allium vineale L. (wild)	608.8±189.5	2.56±0.03	30.67±0.47
Allium sphearocephalum L.	1341.8±372.1	1.29±0.11	50.83±0.73
Allium scorodoprasum L.	1587.6±357.8	25.52±1.209	130.19±1.24
Allium atroviolaceum Boiss.	1376.2±315.3	3.21±0.20	67.09±1.02
Allium cepa L.	473.5±147.4	0.82±0.16	7.61±0.36
Allium pskemenese L.	129.4±86.2	0.07±0.02	7.12±0.77
Allium fistulosum L.	153.4±124.3	0.20±0.02	4.98±0.34
Allium schenoprasum L.	623.4±150.4	4.06±0.24	36.85±0.60
Allium nutans L.	1611.9±619.6	0.30±0.04	12.40±0.16
Allium ursinum L.	108.0±70.7	0.38±0.03	7.27±0.21
Allium flavum L.	639.6±81.5	0.43±0.13	7.74±0.40

Table 3. Quantity of reduced glutathione and contents of flavonoids, vitamin C and soluble proteins in leaves of different *Alliums*

Allium sort	GSH nmol/mg	Flavonoids mg/g	Vitamin C mg/g	Soluble proteins mg/g
Allium sativum L.	0.215±0.009	333.87±12.22	0.036±0,003	2.22±0.09
Allium vineale L. (grown)	0.200±0,003	208.53±4.62	0.253±0.016	4.78±0.23
Allium vineale L. (wild)	0.108±0.001	259.20±0.00	0.066±0.010	4.72±0.12
Allium sphearocephalum L.	0.453±0.004	216.53±4.62	0.811±0.000	1.81±0.05
Allium scorodoprasum L.	2.182±0.016	344.53±9.24	0.142±0.000	0.25±0.03
Allium atroviolaceum Boiss.	0.399±0.011	59.20±13.86	0.157±0.005	3.42±0.05
Allium cepa L	0.127±0.001	496.53±12.22	0.005±0.000	4.78±0.15
Allium pskemenese L.	0.177±0.004	168.53±4.62	0.019±0.003	3.61±0.15
Allium fistulosum L.	0.297±0.008	365.87±9.24	0.061±0.000	7.30±0.15
Allium schenoprasum L.	0.669±0.009	432.53±4.62	0.122±0.000	1.86±0.12
Allium nutans L.	0.125±0.004	53.87±9.24	0.011±0.000	3.68±0.15
Allium ursinum L.	0.148±0.003	171.20±13.86	0.020±0.002	3.97±0.09
Allium flavum L.	0.146±0.002	37.87±9.26	0.344±0.000	4.51±0.04

All measurements were made in triplicate. The values are expressed as mean ± standard error.

Results and discussion

The results obtained from the study are presented in Tables 1, 2, 3. and 4. The SOD activity was detected in leaves of all investigated *Allium* sorts. It had values between 2.51 U/mg potein in *Allium ursinum* L. and 77.46 U/mg protein in *Allium schcnoprasum* L.. C-ase activity ranged from 3.16 U/mg protein (*Allium pskemense* L.) to 23.77 U/mg protein (*Allium vineale* L. grown), P-ase activity from 4.87 U/mg protein (*Allium pskemense* L.) to 130.10 U/mg protein (*Allium scorodoprasum* L.) and GP-ase activity from 0.018 U/mg protein (*Allium nutans* L.) to 0.977 U/mg protein (*Allium scorodoprasum* L.; Table 1). Our results showed that leaves do to various enzyme activities had different susceptibility to the action of toxic oxygen species.

The results obtained from the study of oxygen radicals and MDA are presented in Table 2. The highest $O^{\bullet}_2^-$ quantity was obserwed in leaves of *Allium schorodoprasuu* L. (1587.6 nmol/mg protein) and lowest in leaves of *Allium ursinum* L. (108.0 nmol/mg protein). Quantity of $^{\bullet}OH$, was also highest in leaves of *Allium schorodoprasum* L. (25.52 nmol/mg protein) where the MDA quantity was the highest (130.19 nmol/mg protein) due to toxic oxygen radicals action. The quantity of $O^{\bullet}_2^-$ was lowest in leaves of *Allium psekemense* L. (0.07 nmol/mg protein) and MDA quantity in leaves of *Allium fistulosum* L. (4.98 nmol/mg protein).

The results obtained from the study of the nonenzymic antioxidants and proteins are presented in Table 3. Quantity of GSH ranged from 0.108 nmol/mg protein (*Allium vineale* L. wild) to 2.182 nmol/mg protein (*Allium scorodoprasum* L.). Content of flavonoids ranged from 37.87 mg/g (*Allim flavum* L.) to 496.53 mg/g (*Allium cepa* L.), vitamin C from 0.005 mg/g (*Allim cepa* L.) to 0.811 mg/g (*Allium sphaerocephalum* L.) and soluble proteins from 0.25 mg/g (*Allim scorodoprasum* L.) to 4.78 mg/g (*Allium vineale* L. grown and *Allium cepa* L.).

The content of pigments is presented in Table 4. In leaves of *Allium pskemense* L. the contents of all investigated pigments were the lowest: 0.39 mg/g for chlorophyll a, 0.11 mg/g for chlorophyll b and 0.66 mg/g for carotenoids. The highest content of investigated pigments was obserwed in leaves of *Allium ursinum* L.: 2.87 mg/g for chlorophyll a, 1.35 m/g for chlorophyll b and 9.99 mg/g for carotenoids (Table 4).

Table 4. Pigments content in leaves of different *Alliums*.

Allium sort	Chlorophyll a (mg/g)	Chlorophyll b	Carotenoids
Allium sativum L.	1.64±0.00	0.58±0.00	2.57±0.00
Allium vineale L. (grown)	1.91±0.013	0.94±0.05	3.04±0.01
Allium vineale L. (wild)	0.97±0.017	0.41±0.06	1.30±0.03
Allium sphearocephalum L.	1.08±0.003	0.57±0.06	1.91±0.02
Allium scorodoprasum L.	1.28±0.00	0.70±0.00	0.25±0.01
Allium atroviolaceum Boiss.	0.41±0.01	0.12±0.00	0.71±0.01
Allium cepa L.	1.23±0.05	0.67±0.02	1.92±0.01
Allium pskemanese L.	0.39±0.03	0.11±0.03	0.66±0.01
Allium fistulosum L.	1.14±0.03	0.58±0.03	0.87±0.03
Allium schenoprasum L.	1.48±0.01	0.44±0.03	2.14±0.01
Allium nutans L.	1.65±0.02	0.80±0.07	2.24±0.01
Allium ursinum L.	2.87±0.03	1.35±0.01	9.99±0.01
Allium flavum L.	0.70±0.03	0.33±0.06	1.10±0.01

Our results indicated that some of grown *Alliums* such as *Allium sativum* L., *Allium vineale* L., *Allium cepa* L., *Allium fistulosum* L. and *Allium nutans* L. possessed high antioxidant activities. They had small quantities of $^{\bullet}OH$ and MDA. At the some time SOD, or P-ase activity were high and the content of flavonoids and carotenoids was also high what contribute to their antioxidant abilities.

Among the wild *Alliums, Allium flavum* L. and particulary *Allium ursinum* L. exibited high antioxidant activities. *Allium ursinum* L. had extraordinary antioxidant abilities inspite lowe SOD activity due to high C-ase activity high content of chlorophylls a and b and carotenoids. In leaves of *Allium ursinum* L. small quantities of $O^{\bullet}_2^-$ and $^{\bullet}OH$ were detected what provoced small lipid peroxidation.

Our results indicates that leaves of menshioned grown and wild *Alliums* could be used as a source of natural untoxic antioxidants in food, cosmetic and pharmaceutical industries.

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SYMPOSIUM

Free radical properties of metal complexes+

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ABSTRACT Since metals are cable of catalysing the free radical reactions, different metal (Mg, Mn, Zn) compounds were studied to determine their hydroxyl radical generating and/or scavenging abilities depending on ligand in a H_2O_2/•OH -luminol system with or without microperoxidase. Magnesium oxide, magnesium gluconate and manganese gluconate increase the free radical reactions in the H_2O_2/•OH -luminol system. Most of examined metal compounds have different hydroxyl radical scavenging activity in H_2O_2/•OH- microperoxidase -luminol system. Magnesium citrate has the highest antioxidant effect, while manganese compounds seem to be prooxidant. Vitamin E and C elevate the free radical level in this experimental system.
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KEY WORDS

chemiluminescence
free radical generating ability
scavenging activity
magnesium, manganese and zinc compounds

In living organisms free radical formation and the free radical defense system are closely related to metal ions and complexes (Tampo and Yonaha 1992; Sigel and Sigel 1999). Fenton reaction describes Fe(II) induced hydroxyl radical formation (Fenton 1894, Haber and Willstätter 1931, Haber and Weiss 1934) and other transition metal ions (*e.g.* V, Cr) take part in Fenton-like reactions (Halliwell and Gutteridge 1990; Goldstein et al. 1993). Nevertheless, none transition metal ions also play a significant role, directly or indirectly, in free radical reactions. For example manganese, zinc and copper have a role in the antioxidant defense system (Zidenberger-Cherr and Keen 1991; Ebadi et al. 1996) and magnesium is present in every cell membrane in membrane ATP-ase and magnesium activates several enzymes which catalyse polyphosphate hydrolysis (Fox et al. 2001).

In the diseases where metal ion supplementation is neccessery, it is important to know the complex form (ligand) of the metal. The absorption of metal and other physicochemical properties can change, depending on the ligand (Hallberg et al. 1970; Kaltwasser et al. 1987; Rauch et al. 1990; Szentmihályi et al. 1998) and the physiological effect of the metal compound varies depending on the ligand as well.

Therefore, the hydroxyl radical generating ability and scavenging activity of some metal (Mg, Mn, Zn) compounds were studied by chemiluminescence method in the H_2O_2-luminol system with or without microperoxidase.

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+In memory of Profesor Béla Matkovics

Materials and Methods

The weight ratio of vitamin E to vitamin C was 1:2, and that of vitamins to metals was 2:1 in the experimental system. Concentrations of the metal compounds in the basic solutions were 1 mg/ml. The compounds examined were of pharmacopoeal quality. Since the result is matrix dependent, the measurements were always made under same conditions (temperature, pH, concentration ratios).

Chemiluminescence techniques were applied for the studying of metal catalysis or scavenger activity. Light emission of luminol was measured by a method of Blázovics et al. (1999) using a Berthold Lumat LB-9501 luminometer. The intensity of chemiluminescence light is given as the relative light unit (RLU).

The reaction mixture for measuring free radical generating ability was as follows: hydrogen peroxide (10^4 dilution) 300 μl, luminol ($7x10^{-7}$ M) 100 μl, the sample (1 mg metal compound/ml solution) 50 μl and bidistilled water 250 μl.

The reaction mixture for measuring the hydroxyl scavenging activity was: hydrogen peroxide (10^4 dilution) 300 μl, microperoxidase ($3x10^{-7}$ M) 300 μl, luminol ($7x10^{-7}$ M) 50 μl and the sample (1mg/ml) 100 μl diluted with bidestilled water to 1 ml. The intensity of chemiluminescence light is given as the relative light unit (RLU) reduced by free radical scavenging substances.

Results and Discussion

Since the decomposition of luminol in the presence of hydrogen peroxide is catalysed by metal ions, some metal compounds were investigated for the catalytic activity of the reaction.

Table 1. Free radical generating or scavenging abilities of metal compounds (RLU %) and standard deviation (SD %) in the H_2O_2/•OH- luminol system.

Samples	RLU %	SD %
Background int	100	
Magnesium oxi	125	1.2
Magnesium chl	71.0	0.8
Magnesium sul	20.6	0.4
Magnesium cit	5.1	1.0
Magnesium glu	125	1.1
Manganese dic	26.8	1.3
Manganese sul	12.5	0.3
Manganese glu	102	1.5
Zinc chloride	4.9	0.6
Zinc sulfate	6.6	0.2
Zinc citrate	13.5	1.7
Zinc gluconate	6.0	1.4
Vitamin E and C	15.6	0.5

Table 2. Free radical generating or scavenging abilities of metal compounds (RLU %) and standard deviation (SD %) in the H_2O_2/•OH- microperoxidase-luminol system.

Samples	RLU %	SD %
Background int	100	
Magnesium oxi	177.4	0.9
Magnesium chl	104.7	0.9
Magnesium sul	106	0.3
Magnesium cit	2.3	2.5
Magnesium glu	388	4.9
Manganese dic	364	2.5
Manganese sul	366	0.4
Manganese glu	203	3.0
Zinc chloride	201.6	1.1
Zinc sulfate	82.6	0.1
Zinc citrate	74.3	3.6
Zinc gluconate	82.1	6.6
Vitamin E and	91.6	0.4

Table 3. Free radical generating or scavenging abilities of metal compounds in the presence of Vitamin E and C (RLU %) and standard deviation (SD %) in the H_2O_2/•OH- microperoxidase-luminol system

Samples	RLU %	SD %
Background intensity	100	
Magnesium sulfate	191.5	1.2
Magnesium gluconate	182.9	0.8
Manganese sulfate	240	0.3
Manganese gluconate	160.5	0.4
Zinc sulfate	95.9	0.1
Zinc gluconate	94.4	0.4

In the applied system and concentrations neither vitamins nor metal compounds (except for magnesium oxide, magnesium gluconate and manganese gluconate) generated the decomposition of luminol to aminophtalate in H_2O_2/•OH-luminol system (Table 1). When we applied the microperoxidase in the system (H_2O_2/•OH-microperoxidase-luminol) different metal compounds have different radical scavenging activity (Table 2). The metal ions, depending on the quality of the metal, ligand and concentration, may induce free radicals. Among magnesium compounds, only magnesium citrate has significant scavenging activity whereas other magnesium compounds enhance the free radical level formation. None of the manganese compounds have hydroxyl radical scavenging activity, while zinc compounds (except for zinc chloride) show scavenging activity. The mixture of vitamin E and C in the applied concentration has only a weak scavenging activity in this system.

The presence of vitamin E and C in the metal ion-containing solution changes the free radical scavenging activity. The vitamins increased the relative light unit (RLU %) of the system (H_2O_2/•OH-microperoxidase-luminol) together with magnesium sulfate, zinc sulfate and zinc gluconate solutions, while decreased free radical formation together with magnesium gluconate, manganese sulfate and manganese gluconate solutions (Table 3).

According to the results it has been stated that chemiluminescence measurement is an efficient method for the determination of free radical generating or scavenging activity of metal compounds as well.

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SYMPOSIUM

Antioxidant effect of various rosemary (*Rosmarinus officinalis* L.) clones+

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ABSTRACT The antioxidant effects, the levels of total phenol and the total phenol contents of volatile oils and plant extracts were determined in eight various Rosemary (*Rosmarinus officinalis* L.) clones. Antioxidant acitvities and the total phenol contents were measured by spectophotometric method as well as the volatile oil content of the fresh plants with gas chromatograph. Our preliminary results clearly indicate that the antioxidant capacity of volatile oils and plant extracts closely related to the total phenol contents. Reason of the observed differences should be revealed by the determination of the quantity and quality of the individual volatile oil components.
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KEY WORDS

Rosmarinus officinalis L.
rosemary oil
total phenol content
FRAP

Rosemary (*Rosmarinus officinalis* L.) is a very important medicinal and aromatic plant, which belongs to the Lamiaceae family and has been cultivated for a long time. Anthropologists and archaeologists have found evidence that rosemary herbs were used as medicinal, culinary and cosmetic virtues in the ancient Egypt, Mesopotamia, China and India.

Rosemary is a widely used aromatic and medicinal plant nowadays. *Rosmarini folium* has antibacterial, antioxidant and antiphlogistic effect. The essential oil enhances the blood-circulation of the limbs, has antirheumatic effect and relieves the neuralgic pains. Besides the therapeutical application, the essential oil is widely applied in the cosmetic industry producing various Cologne waters, bathing essences, hair lotions and shampoons. The leaf of rosemary is an indispensable spice of the French, Italian and Spanish cuisine.

Rosemary is a perspective plant culture in the world, it is in the middle of interest of plant breeders (Chalchat et al. 1993; Domokos et al. 1997; Mulas et al. 1998). Because of its sensitivity to cold rosemary was not cultivated in Hungary, until a frost-tolerant cultivar of rosemary the "Harmat" (Domokos et al. 1997) was not isolated.

Rosemary is cultivated for the valuable oil which can be extracted from the harvested plants when flowers are in buds.

It is well known that the activity of rosemary extracts in food industry and medicine due to the presence of some important antioxidant oil and phenolic components (Cuvelier et al. 1996; Fadel and El-Massry 2000), to prevent oxidative degradation of oil and lipid containing foods (Economou et al. 1991; Banias et al. 1992; Chen et al. 1992; Clifford and Cuppet 1993; Pokorny 1997). Its antioxidant properties not only exploited by the food industry but by the plant protection techniques and therapy (Mongold et al. 1991; Paris et al. 1993), as well.

Our aim was to compare oil contents as well as the level of total phenol and antioxidant effects in the volatile oil and in the plant extracts of different originated rosemary clones.

Materials and Methods

Plant material (*Rosmarinus officinalis* L.) of the experiment were originated from rosemary clones of the germplasm collection of the Department's Research Station, Soroksár. Fresh plant materials (160 mg/10 ml) were extracted with methanol : water 4:1 for 2 hours (Table 1).

The essential oil content of the leaf drug was determined by hydrodistillation applying improved Clevenger-type apparatus, according to the Hungarian Pharmacopoeia VII at the laboratory of the Department of Medicinal and Aromatic Plants.

Table 1. Origins and marks of the samples.

Marks	Origin	Sample
A	Hungary	Harmat
B	Italy	Blauer Toscaner
C	Croatia	Horváth
D	Morocco	Salem
E	Germany	Veitschöchheim
F	Spain	Majorca
G	Greece	Gorizia
H	England	Hardy

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+In memory of Professor Béla Matkovics

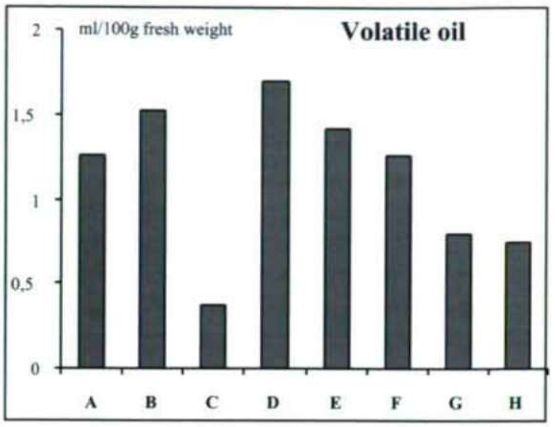


Figure 1. Volatile oil contents (ml/100 g fresh weight) of various rosemary (*Rosmarinus officinalis* L.) clones.

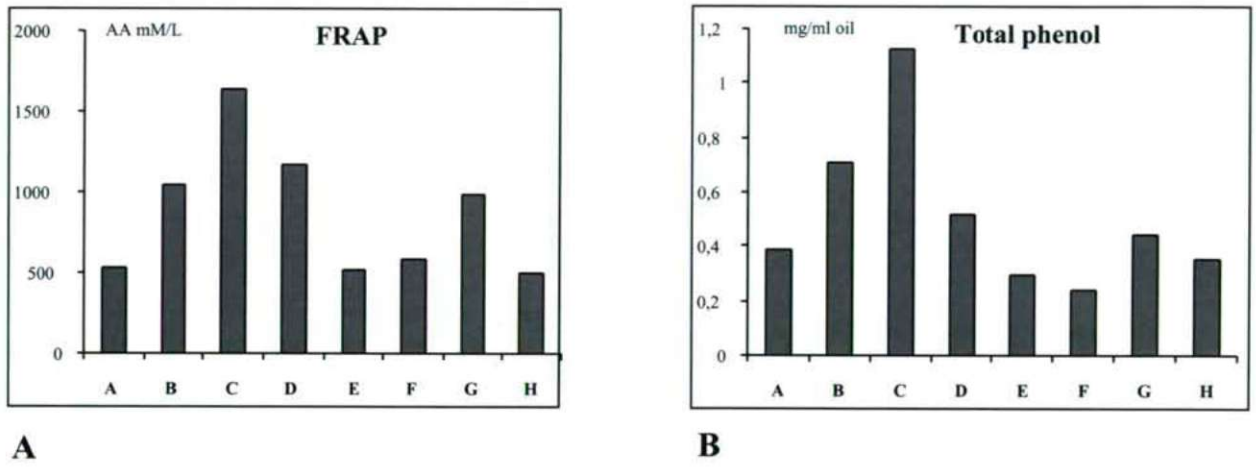
Total soluble phenols were determined using Folin-Ciocalteu reagent according to the method of Singleton and Rossi (1965). The content of soluble phenols was calculated from a standard curve obtained with different concentrations of gallic acid.

Antioxidant power was measured by the FRAP (Ferric Reducing Ability of Plasma) method at λ=593 nm (Benzie and Strain 1996).

Results and Discussion

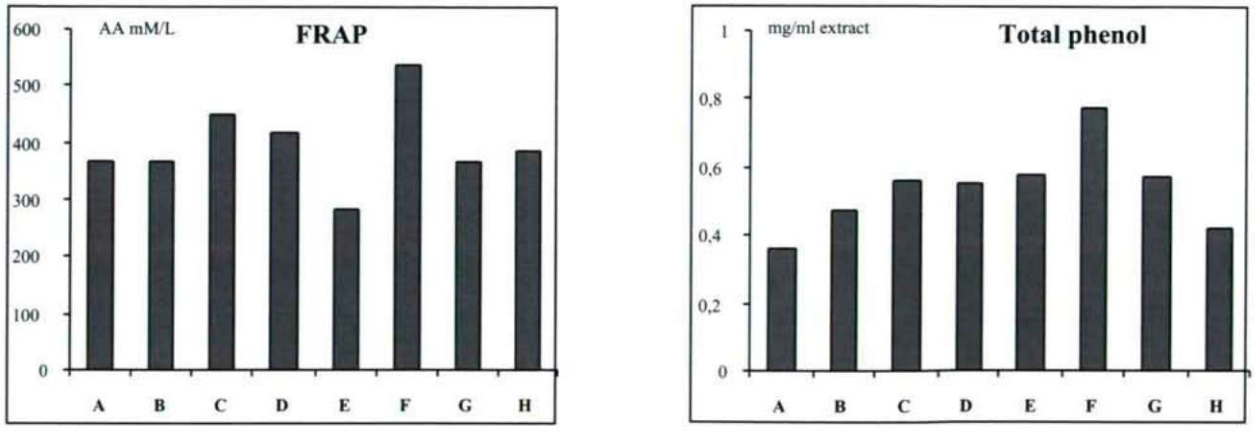
Our results are summarized in Figures 1-3. We have found differences in the volatile oil contents of the samples ranging from 0.368 to 1.691 ml oil/ 100g fresh weight (Fig. 1).

Marked differences occurred in the FRAP values of volatile oils (Fig. 2/A). Threefold differences were detected between the lowest and the highest level of the ascorbic acid



A B

Figure 2. Antioxidant capacity (A) and total phenol content (B) in oils of various rosemary (*Rosmarinus officinalis* L.) clones.



A B

Figure 3. Antioxidant capacity (A) and total phenol content (B) in plant extracts of various rosemary (*Rosmarinus officinalis* L.) clones.

(AA) equivalent antioxidant activities. The "Horvát" (C) clone showed the highest FRAP-values (1643 AA mM/L).

We have found similar tendency in the case of total phenol contents, the "Horvát" (C) clone showed the highest level (1.12 mg/ml oil).

In contrast to the above mentioned results, plant extract of "Majorca" (F) possessed the highest antioxidant capacity and total phenol content (Fig. 3).

Our preliminary results clearly indicate that the antioxidant capacity of volatile oils and plant extracts closely related to the total phenol contents. Reason of the observed differences should be revealed by the determination of the quantity and quality of the individual volatile oil components.

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SYMPOSIUM

Examination on antioxidant activity in the greater celandine (*Chelidonium majus* L.) extracts by FRAP method+

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ABSTRACT Antioxidant activity in the alcoholic extracts (20 and 40%) of the greater celandine (*Chelidonium majus* L.) herb was investigated by FRAP (ferric reducing and antioxidant power) method. Since the antioxidant activity of the extracts greatly depends on the quality of compounds, the phytochemical examination for alkaloid- and element content were also examined. According to the results the antioxidant activity does not depend on the alkaloid content of the drug during the vegetation period and on the alkaloid content of the alcoholic extracts. It seems that the antioxidant activity of the extracts is also independent from the transition metal element content.

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KEY WORDS

Chelidonium majus L.
alcoholic extract
FRAP method
alkaloid content
metal ion content

The greater celandine (*Chelidonium majus L.*) is not listed in the Hungarian Pharmacopoeia in spite of the fact that the drug is commercially available in the herb-trade. In folk medicine the antiviral activity of the plant is attributed to its alkaloids present in the freshly outflowing latex (Dickson 1996). The alkaloid components of the orange coloured latex of the plant (chelidonine, chelerythrine, coptisine, sanguinarine, berberine etc.; Then et al. 2000) also have a number of beneficial effect, *e.g.* spasmolytic-, antiinflammatory-, antimicrobial-, antiviral-, antifungal-, antitumor activity and cytotoxic properties (Khayyal et al. 2001; Coon and Ernst 2002; Kokoska et al. 2002).

The antioxidant activity of the drug and extracts has not examined yet. Therefore, the antioxidant activity was measured by FRAP method and evaluated on the bases of alkaloid and element content. The the FRAP method means the ferric reducing ability of plasma or plants (Benzie and Strain 1996, 1999). Ferric to ferrous ion reduction at low pH causes a ferrous-tripyridyl-triazine complex which has absorbtion at 593 nm. FRAP values are obtained by comparing the absorbance changes at the given vawelength and how influence the added plasma aliquots the FRAP values. Absorbance changes are linear over a wide concentration range with antioxidant mixture, including plasma or purified antioxidant mixture. The known antioxidants are interact very much. The FRAP assays are inexpensive, simple to prepare the reagents, the results are highly reproducible and measurement takes no long time.

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+In memory of Professsor Béla Matkovics

Materials and Methods

Fresh aerial parts of greater celandine (*Chelidonium majus L.*) were collected from the Botanical Garden of Budapest in 2002.

I. Extraction: The plant (5 g) was poured with alcoholic water (100 ml, 20 and 40%, 60°C) and allowed to stand at room temperature for 24 hours, then filtered.

II. Extraction for antioxidant activity of drug sample: The plant (1.5 g) was poured with double distilled water (200 ml) and allowed to stand at room temperature for 30 min, then filtered.

For the determination of total alkaloid content of plant and extracts, the reference method chosen was the measurement of chelidonine content according to the German Pharmacopoeia (DAB 10) as follows. The plant (0.75g) or the solution (25 ml) was extracted with CH_3COOH (200 ml, 12%, g/v) by refluxing on a water bath for 30 min. After cooling the solution was filtered into a volumetric flask (250 ml). The acetic acid extract (30 ml) was made alkaline (pH 8-9) with NH_4OH (25%) then extracted with $CHCl_3$ (3x30 ml) in a separatory funnel. The $CHCl_3$ phase was mixed and dried on anhydrous Na_2SO_4 and after filtration, $CHCl_3$ was evaporated under vacuum. The residue was redissolved in CH_3CH_2OH (2.5 ml) and transferred into a volumetric flask (25 ml). The trace residue was washed with diluted H_2SO_4 (10%, 3x5 ml) and also transferred into the volumetric flask, then the solution was diluted to 25 ml. A mixture of this solution (5 ml) and chromotropic acid (5 ml) was diluted with H_2SO_4 (98%) to 25 ml, then kept on a boiling water bath (100°C) for 10 min. After cooling, the absorption of the solution was measured at 570 nm against

Table 1. Alkaloid content (%, g/100 g) and antioxidant activity (μmol/l) of the greater celandine (*Chelidonium majus* L.) plant herb during the vegetation period.

Harvested period	Alkaloid content (%)	Antioxidant activity (μmol/L)
III. month	0.218 ±0.023	569.5 ±9.41
IV. month	0.196 ±0.015	289.5 ±018.5
VI. month	0.117 ±0.008	422.2 ±32.1

Table 2. Alkaloid content (%, g/100 ml) and antioxidant activity (μmol/l) in the alcoholic extracts of the greater celandine (*Chelidonium majus* L.).

Samples	Alkaloid content (%)	Antioxidant activity* (μmol/L)
20 % alcoholic extract	0.172 ±0.008	90.6 ±9.4
40 % alcoholic extract	0.380 ±0.009	91.4 ±15.2

*20 % and 40 % alcoholic-aqueous solution have no antioxidant activity.

Table 3. Element concentration (μg/ml) in the alcoholic extracts of the greater celandine (*Chelidonium majus* L.).

Elements	0 % alcoholic extrac	0 % alcoholic extract
Ca	10.94 ±0.29	6.25 ±0.20
Cu	0.273 ±0.173	0.188 ±0.074
Fe	0.217 ±0.023	0.137 ±0.029
K	52.36 ±5.07	67.94 ±0.17
Mg	17.01 ±0.63	11.90 ±0.12
Mn	0.141 ±0.009	0.086 ±0 008
Na	39.21 ±0.80	23.50 ±0.20
P	30.41 ±0.27	19.83 ±0.15
Zn	0.464 ±0.006	0.346 ±0.006

the blank solution. The extinction coefficient in 1% solution in a 1 cm vessel was 933 (100 ml g^{-1} cm^{-1}).

The FRAP method for measuring the ferric reducing ability of plasma (FRAP) or plants (Benzie and Strain 1996, 1999) is the following:

Reagents:

1. Acetate buffer, 300 mmol/L pH 3.6 (3.1 g sodium acetate x3H_2O and 16 ml acetic acid in 1000 ml buffer solution).

2. 10 mmol/l 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mmol/l HCl.

3. 20 mmol/l $FeCl_3$x6H_2O in distilled water.

FRAP working solution: 25 ml acetate buffer (1), 2.5 ml TPTZ solution and 2.5 ml $FeCl_3$x6H_2O solution.The working solution must be always freshly prepare.

Aqueous solution of known $FeSO_4$x7H_2O was used for calibration.

Assay: Blank: FRAP reagent.

Sample: FRAP reagent 1,5 ml and sample solution 50 μl.

Monitoring up to 5 min at 593 nm, 1 cm light path and 37°C. Fe(II) standard solution tested in parallel. Calculation: usig the calibration curve.

Element concentration of samples was determined by an inductively coupled plasma atomic emission spectrometer (ICP-AES). Type of instrument: Atom Scan 25 (Thermo Jarrell Ash), a sequential emission spectrometer. Sample handling: the samples (50 ml of evaporated extract) were digested with a mixture of HNO_3 (5 ml) and H_2O_2 (3 ml) in teflon vessels. After digestion the samples were diluted to 25 ml, from which the following elements were determined in three parallel measurements: Ca, Cu, Fe, K, Mg, Mn, Na, P, Zn.

Results and Discussion

The drug samples was collected during the vegetation period in different time to observe the changes of the antioxidant activity with total alkaloid content. Total alkaloid content of the samples was measured accordance with the German Pharmacopoeia (DAB 10), and the antioxidant activity was assayed by FRAP method (Table 1). According to the results we stated that the alkaloid content of the drug changes during the vegetation period and it seems that the antioxidant activity does not depend on the alkaloid content of the drug.

Alcoholic extracts (20 and 40%) of the greater celandine contains different amount of alkaloids with almost the same and very low antioxidant activity (Table 2). This result confirms our previous measurements obtained during vegetation period. Alcoholic solutions (20 and 40%) do not have an influence on the antioxidant activity.

The extracts contain elements as well dissolved in or bound by organic compounds (Buzuk et al. 2001). Element content of the extracts is very low (Table 3) and they do not contain transition metal ions in higher concentration than other alcoholic extracts (Szentmihályi and Then 2001; Szentmihályi et al. 2001).

Though the greater celandine (aqueous extract) has antioxidant activity, this value semms to be independent from its alkaloid and element content.

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SYMPOSIUM

The role of antioxidant phytonutrients in the prevention of diseases*

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ABSTRACT The authors investigated the *in vitro* antioxidant properties of nine selected flavonoid aglycons, namely quercetin, kaempferol, myricetin, apigenin, luteolin, daidzein, genistein, formononetin and biochanin A. The *in vitro* antioxidant power of flavonoids basically depends on their chemical structure. The concentration of those flavonoids in vegetables and fruits frequently consumed in Hungary was examined by a RP-HPLC method. Referring to the total flavonoid content, rich sources were onions, parsnip, spinach, different parts of celery, and lentils. Among the fruits berries were very rich sources of flavonoids (blackberry, red current). The flavonoid intake was estimated in two groups, first included more than 500 schoolboys and girls aged 12-15 years, and the second group was about 200 healthy adults aged 25-60 years. The average dietary flavonoid intake of the children and the adults was 19.5±26.6 and 18.8±28.9 mg/day, respectively. The flavonoid intake showed high differences among the subjects. The consumption of flavonoids in the children's group was from 0 to 179 mg/day, and in the adults¢ group from 0.5 to 310 mg/day. Although two groups in our study did not represent exactly the total Hungarian population it is supposed that the average daily flavonoid intake of the population is not different from these data. The estimated Hungarian intake is very similar to that of others published in the literature.
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KEY WORDS

flavonoids
antioxidant properties
plant foods
dietary intake

The antioxidant characteristics of plant derived materials can be attributed to their content of polyphenols. Until recently, most of the nutritional interest in polyphenols was in the deleterious effects caused by the ability of polyphenols to bind and precipitate macromolecules, such as dietary protein, carbohydrate, and digestive enzymes, and therefore reducing food digestibility. However, interest in food phenolics has increased, because of their antioxidant and free radical scavenging abilities. Polyphenols constitute one of the most numerous and widely distributed group of substances in the plant kingdom, with more than 8000 phenolic structures currently known (Harborne 1993). Polyphenols are products of secondary metabolism of plants and ubiquitous in all plant organs. They arise biogenetically from two main synthetic pathways: the shikimate and the acetate pathway (Harborne 1993; Bravo 1998).

Natural polyphenols can range from simple molecules, such as phenolic acids, to highly polymerised compounds, such as tannins. They occur primarily in conjugated form, with one or more sugar residues linked to hydroxyl groups, although direct linkages of the sugar unit to an aromatic carbon atom also exist. According to Harborne (1989) polyphenols can be divided into at least 10 different classes depending on their basic chemical structure. Flavonoids, which constitute the most important single group, can be further subdivided into 13 classes, with more than 4000 compounds described until 1990 (Harborne 1989, 1993). Compounds most widely occurred in the nature are flavonols, flavones, flavan-3-ols, isoflavones, flavanones, flavanols, anthocyanidines and proanthocyanidines (Bravo 1998).

Flavonoids are almost ubiquitous in plant foods (vegetables, cereals, legumes, fruits, nuts, etc.) and beverages (wine, cider, beer, tea, cocoa, etc.). The presence of flavonoids in plant foods is largely influenced by genetic factors and environmental conditions. Other factors such as germination, degree of ripeness, variety, processing, and storage also influence the content of plant phenolics (Herrman 1976, 1988; Mazza 1995; Peleg et al. 1991). Flavonoids and other polyphenols are partially responsible for sensory and nutritional qualities of plant foods. The astringency and bitterness of foods and beverages depends on the content of polyphenols.

Many flavonoids and polyphenols can exhibit antioxidant activity as their extensive, conjugated π-electron systems allow ready donation of electrons, or hydrogen atoms, from the hydroxyl moieties to free radicals (Bors et al. 1987). However, the antioxidant efficacy, in terms of reaction stoichiometry (the number of radicals which one phenolic molecule may annihilate) and reaction kinetics (the rate at

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which radicals are annihilated), may vary considerably. This will depend on structural features, such as the number and positions of the hydroxyl moieties on the ring systems, and the extent by which the unpaired electron in the oxidised phenolic intermediate can delocalise throughout the molecule. Most polyphenols, especially flavonoids are very effective scavengers of hydroxyl and peroxyl radicals (Manach et al. 1996). Flavonoids are chelators of metals and inhibit the Fenton and Haber-Weiss reactions, which are important sources of active oxygen radicals (Shahidi and Wanasundara 1992). In addition, flavonoids retain their free radical scavenging capacity after forming complexes with metal ions (Afanas'ev et al. 1989).

Interest in food phenolics has increased owing to their role as antioxidants, antimutagens, and scavengers of free radicals and their implication in the prevention of pathologies such as cancer and cardiovascular disease. Epidemiological studies have shown a correlation between an increased consumption of phenolic antioxidants and a reduced risk of cardiovascular disease and certain types of cancer (Hertog et al. 1993, 1995; Hertog 1996; Rimm et al. 1996; Hollman and Katan 1999). Food products from fruits and vegetables are important part of a well-balanced, healthy diet in humans.

Flavonols, flavones and isoflavones constitute three major subclasses of flavonoids (Fig 1). Flavonols and flavones have similar C-ring structure with a double bond at the 2-3 position. Flavones, as opposed to flavonols, lack a hydroxyl group at the 3-position. Major flavonols are quercetin (3,5,7,3',4'-pentahydroxyflavone), kaempferol (3,5,7,4'-tetrahydroxyflavone), and myricetin (3,5,7,3',4',5'-hexahydroxyflavone). The most abundant flavones in plants are luteolin (5,7,3',4'-tetrahydroxyflavone) and apigeo=n (5,7,4'-trihydroxyflavone). B-ring of isoflavonoids is connected to 3-C. Most frequently occurred isoflavonoids are isoflavones with a double bond between 2- and 3-C. Major isoflavones are genistein (5,7,4'-trihydroxyisoflavone), daidzein (7,4'-dihydroxyisoflavone), formononetin (7-hydroxy-4'-methoxyisoflavone) and biochanin A (5,7-dihydroxy-4'-methoxyisoflavone).

In present study the *in vitro* antioxidant properties of some selected flavonoids were investigated, the flavonoid composition of plant foods was measured, and based on these analytical data the flavonoid intake in two groups of the Hungarian population was estimated.

Materials and Methods

Chemicals

Quercetin, luteolin, myricetin, kaempferol, genistein, daidzein, formononetin, biochanin A and t-butylhydroquinone were purchased from Sigma, apigenin from Fluka and methanol of chromatography grade were obtained from Merck. All other chemicals and reagents were of analytical grade from Reanal (Hungary).

Fresh and dried fruits

45 selected fresh and dried fruits and 31 vegetables (1 kg, or a minimum of three units) were purchased from 3 different greengrocers in the local markets in Budapest at a period of their most frequent consumption. The edible parts of the fruits and vegetables were used to the examination, and samples from three locations were combined. After buying the samples were immediately cleaned, chopped into small pieces and freeze-dried. After lyophilization, samples were allowed to equilibrate in open air and ground to pass a 0.5-mm sieve. Moisture was measured by drying at 105°C. The food samples were stored at -18°C for less than 4 months until analysed.

Hydrogen-donating ability

Hydrogen-donating ability of pure flavonoids dissolved in methanol was determined in the presence of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as described Blois (1958) and modified by Hatano et al. (1988). Hydrogen-donating ability is expressed as I_{50}, the amount of the sample that is needed for 50% discoloration of DPPH. The lower the value the higher the activity.

Reducing power

Reducing power of methanolic solution of flavonoids was determined according to the method of Oyaizu (1986). Diluted and/or filtered sample (1 ml) was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 1% potassium ferricyanide (2.5 ml); the mixture was incubated at 50°C for 20 min. Trichloracetic acid (2.5 ml, 10%) was added to the mixture which was then centrifuged at 1.500 g for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and freshly prepared $FeCl_3$ solution (0.5 ml, 0.1%). The absorbance was read at 700 nm. Increased absorbance of the reaction mixture indicates elevated reducing power. Reducing power is given in ascorbic acid equivalent (ASE ml^{-1}) that shows the amount of ascorbic acid expressed in mmol those reducing power is the same than that of 1 ml sample.

Total antioxidant status

This spectrophotometric technique measures the relative abilities of antioxidants to scavenge the $ABTS^{\cdot+}$ [2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate)] in comparison with the antioxidant potency of standard amounts of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). The measurement is based on the procedure described by Miller et al. (1993). The radical cation $ABTS^{\cdot+}$, produced by the ferrylmyoglobin radical generated from methmyoglobin and H_2O_2 in the presence of peroxidase, is a blue-green chromogen with characteristic absorption at 660 nm. In the presence of antioxidants absorbance is decreased.

The determination of total antioxidant status (TAS) of flavonoids was carried out using the Randox diagnostic kit with a COBAS MIRA automatic laboratory analyser.

HPLC analysis of flavonoids

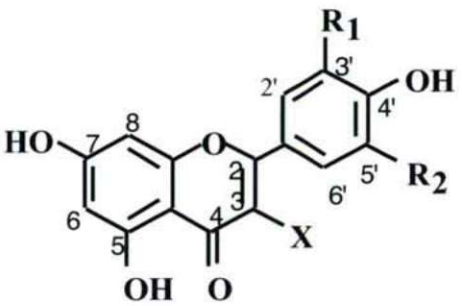
The flavonols (quercetin, kaempferol, myricetin) and the flavones (apigenin, luteolin) were measured as aglycons according to Hertog et al. (1992). Briefly, flavonoid glycosides were extracted and hydrolysed to their aglycons with 2.0 M HCl in boiling 50% aqueous methanol in the presence of 0.1 g t-butylhydroquinone for two hours. After refluxing the extract was allowed to cool and was subsequently made up to 50 ml with methanol and sonicated for 5 min. Approximately 2 ml was filtered through 0.45 μm filter (Chromafil AO-20/25) before injection. The resulting aglycons were quantified by RP-HPLC (Perkin Elmer) on a Premisphere C_{18} column (150 x 3.9 mm, 5 μm, Phenomenex, USA) using methanol/phosphate buffer (45/55 v/v, pH 2.4), as a mobile phase and UV detection (370 nm).

Limit of detection was defined as the amount of flavonoids resulting in a peak height of 3 times higher than the standard deviation of the baseline noise. Peak identification was confirmed with the use of known retention time of pure flavonoids. Quantification of the flavonoids was by peak area measurement. Calibration curves of individual flavonoids were made over a range of 1-8 μg/ml. Detector response was linear over the concentration range used. For all standards r^2 was higher than 0.998.

Estimation of dietary intake of flavonoids

Data of flavonoids come from the analytical results were built into an existing computer system used for calculation of the intake of different nutrients. Based on these data the flavonoid content of more than 2600 meals was calculated. Estimation of flavonoid intake in two groups of the Hungarian population was done according to the international standard method, a three-days dietary record. Persons involved in the study were asked to note everything that they consumed during three different days (2 non-consecutive weekdays, 1 weekend day). Avoiding the incorrect data qualified dieticians helped and interviewed the subjects. The flavonoid intake was estimated in two groups, first included

Flavonoids



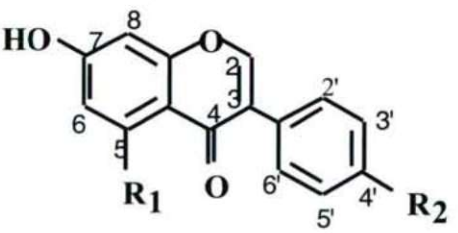
Flavonols: X = OH

quercetin	$R_1 = OH, R_2 = H$
kaempferol	$R_1 = H, R_2 = H$
myricetin	$R_1 = OH, R_2 = OH$

Flavones: X = H

apigenin	$R_1 = H, R_2 = H$
luteolin	$R_1 = OH, R_2 = H$

Isoflavonoids



formononetin	$R_1 = H, R_2 = OCH_3$
daidzein	$R_1 = H, R_2 = OH$
biochanin A	$R_1 = OH, R_2 = OCH_3$
genistein	$R_1 = OH, R_2 = OH$

Figure 1. Chemical structure of flavonoids and isoflavonoids.

Table 1. *In vitro* antioxidant activity of different flavonoids.

Family	Molecule	Hydrogen donating ability[1], I_{50} (mg)	Reducing power[2] (ASE/mg)	TAS[3] (mmol/g)
Flavonols	**Quercetin**	7.3	15.0	92.6
	Myricetin	9.9	15.3	98.7
	Kaempferol	19.0	9.3	8.3
Flavones	Luteolin	7.2	9.9	16.5
	Apigenin	>390	0.73	4.3
Isoflavonoids	Genistein	1441	0.130	8.85
	Daidzein	1588	0.060	1.98
	Biochanin A	672	0.117	2.30
	Formononetin	4254	0.028	1.32

[1]I_{50} expressed in mg is the amount of the molecule that is needed for the 50% discolouration of DPPH radical.
[2]Reducing power is given in ascorbic acid equivalent (ASE) that shows the amount of ascorbic acid expressed in mmol those reducing power is the same than that of 1 ml sample. [3]Total antioxidant status (TAS) is measured by the Randox Kit and the characteristic is expressed in Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent.

521 schoolboys and girls aged 12-15 years, and the second group was 204 healthy adults aged 25-60 years.

Results and Discussion

In vitro antioxidant activity of flavonoids

Flavonol and flavon molecules exhibited strong antioxidant properties in different *in vitro* systems (Table 1). The flavonol quercetin and myricetin, and the flavon luteolin were effective hydrogen-donating molecules in the presence of DPPH radical, because they had low I_{50} value that is the amount of the molecule needed for the 50% discolouration of the DPPH radical. As it can be seen on Figure 1 quercetin and luteolin have two OH groups on B ring, while myricetin has three, and kaempferol and apigenin have only one. Luteolin and apigenin do not have OH group at C-3 position. As Bors and co-workers (1990) established based on their studies with pulse radiolysis three structural elements are essential for the strong antioxidant property of a flavonoids: 1) the *ortho*-dihydroxy (catechol) structure in the B-ring, the obvious radical target site for all flavonoids with saturated 2,3-double bond (flavan-3-ols, flavanones, cyanidin chloride); 2) the 2,3-double bond in conjunction with a 4-oxo function; and 3) the additional presence of both 3- and 5-OH groups for a maximal radical scavenging potential (Fig. 2). Results of reducing power and TAS also reflect these connections between the chemical structure and antioxidant property. In this *in vitro* system quercetin was the most effective antioxidant while apigenin was the weakest compound. For the effective antioxidant property a resonance stable chemical structure is essential as it is realized in the case of quercetin (Fig. 3).

Isoflavonoids also expressed antioxidant properties but these were much lower than those of flavonols and flavones (Fig. 1). B-ring of the isoflavonoids is connected with 3-C instead of 2-C unlike in flavonoids. From isoflavonoids studied here genistein and biochanin were more effective than daidzein and formononetin. It is clear that both hydroxy groups in the 4' and 5 position are needed for antioxidant activity of the molecule as in genistein. Biochanin A have similar activity, OCH_3 group at 4' position do not modify considerably the antioxidant property. According to TAS result genistein is stronger than apigenin but the two other parameters as H-donor ability and reducing power show opposite property. The resonance-stabilized quinoid structures of isoflavones show that the carbonyl group at position 4-C loses its functionality (Shukla et al. 1997) thus explaining the similar or a bit stronger antioxidant activity of genistein compared with apigenin.

Flavonoids and isoflavonoids exhibited significant antioxidant properties in different *in vitro* tests. Antioxidant effectiveness of a compound is significantly dependent on its chemical structure. However, it is very important to take into account that chemical structure of the compounds could dramatically change during metabolism. Functioning of intestinal bacteria and different enzymes, hepatic/microsomal transformations that are hydroxilation, methylation, reduction, conjugation, can lead to a new compound having different antioxidant properties from the original molecule. Therefore the results of *in vitro* studies have to be managed in a circumspect way.

Flavonoid composition of plant foods

Flavonol and flavon content of plant foods is strongly influenced by extrinsic factors such as variation in plant type

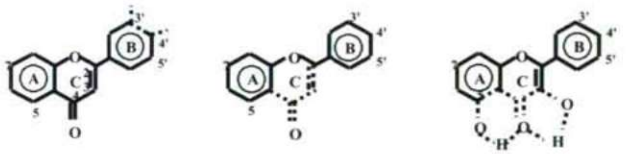


Figure 2. Structural elements of flavonoids responsible for the antioxidant properties.

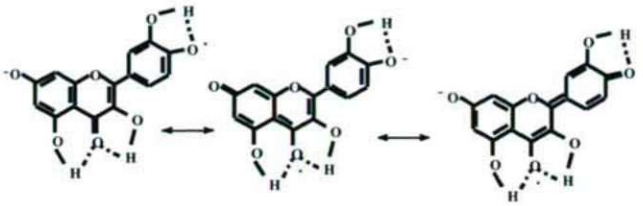


Figure 3. Resonance stable structure of quercetin.

and growth, season, climate, and degree of ripeness (Hollman and Arts 2000; Stewart et al. 2000; Parr and Bolwell 2000). Flavonoid composition of some fruits and vegetables investigated in this study is presented in Table 2. More detailed data are published elsewhere (Lugasi and Hóvári 2000, 2002).

Quercetin and kaempferol are proved to be the most widespread flavonoids in vegetables. Quercetin levels in the edible parts of most vegetables were generally below 10 mg/kg. The highest quercetin concentration could be detected in spinach (272.2 mg/kg) and in the different types of onion (67.1-171.3 mg/kg). High quercetin level was also found in dill (74.5 mg/kg), crisped lettuce (35.0 mg/kg), and broccoli (15.4 mg/kg). Very low level or no quercetin was detected in different root vegetables such as celery and beetroot, radishes and also in *Brassica* vegetables except broccoli. Significant amount of kaempferol was observed in parsnip (66.4 mg/kg), leek (45.8 mg/kg), fresh onion collected in early summer (34.3 mg/kg), and broccoli (30.8 mg/kg). Average level of flavonoid could be detected in other *Brassica* and root vegetables. Practically there was no kaempferol in leafy vegetables except crisped lettuce (8.4 mg/kg). The flavonol myricetin only in five vegetables was found, namely Swedish turnip (85.4 mg/kg), parsley leaves (80.8 mg/kg), celery leaves (43.4 mg/kg), lettuce (10.2 mg/kg), and dill (7.0 mg/kg). Regrettable the leaves of parsley, celery and dill are consumed as a condiment in special Hungarian dishes therefore the participation of these vegetables in the flavonoid intake of the population is probably negligible. Significant amount of flavon luteolin could be detected in celery leaves (111.4 mg/kg), spinach (66.4 mg/kg), red beet (18.3 mg/kg), kohlrabi (13.0 mg/kg), and different types of pepper (10.7-11.3 mg/kg). Apigenin was detected in celery leaves (248.0 mg/kg), Swedish turnip (154.0 mg/kg), and celery root (24.1 mg/kg).

In present investigation all of vegetables had significant amount of flavonols and/or flavones. The best sources of selected five flavonoids were spinach (338.6 mg/kg), Swedish turnip (265.3 mg/kg), red, young and old onions (195.6,

Table 2. Flavonoid content of some plant food (mg/kg fresh weight).

Sample	Quercetin	Kaempferol	Myricetin	Luteolin	Apigenin	Total flavonoids
Vegetables						
Broccoli	15.4	30.8	nd*	nd	nd	46.2
Kohlrabi	4.0	24.3	nd	13.0	nd	41.3
White cabbage	1.6	11.9	nd	4.2	nd	17.7
Red onion	121.5	2.6	nd	nd	nd	124.1
Purple onion	171.3	24.3	nd	nd	nd	195.6
Pepper	9.4	nd	nd	10.7	nd	20.1
Crisped lettuce	35.0	8.4	nd	3.9	nd	47.3
Spinach	272.2	nd	nd	66.4	nd	338.6
Dill	74.5	nd	7.0	nd	nd	81.5
Parsley leaves	nd	nd	80.8	nd	nd	80.8
Celery leaves	nd	nd	43.4	111.4	248	402.8
Celery root	1.8	nd	nd	nd	24.1	25.9
Swedish turnip	3.2	22.7	85.4	nd	154.0	265.3
Horse radish	5.7	25.7	nd	9.0	nd	40.4
Pumpkin	nd	nd	nd	16.3	nd	16.3
Fruits						
Water-melon	nd	nd	nd	18.4	nd	18.4
Musk-melon	nd	nd	nd	25.8	nd	25.8
Sour cherry	29.2	nd	nd	nd	nd	29.2
Mulberry	24.7	nd	452.6	nd	nd	477.3
Blackcurrant	52.8	nd	nd	nd	nd	52.8
Blackberry	14.0	nd	636	nd	nd	650
Strawberry	9.0	nd	994	nd	nd	1003
Grape (Saszla)	38.7	nd	nd	nd	nd	38.7
Walnut	nd	nd	4565	nd	nd	4565
Kiwi	nd	nd	nd	22.3	nd	22.3
Banana	nd	nd	22.8	nd	nd	22.8
Apple	38.3	nd	nd	27.0	nd	65.3
Pear	24.7	nd	nd	nd	nd	24.7
Plum	23.3	nd	nd	nd	nd	23.3
Apricot	11.5	nd	nd	nd	nd	11.5

*nd – under the detection limit

124.1, and 101.6 mg/kg, respectively), and celery leaves (154.8 mg/kg). As the formation of flavonoids is light-dependent, flavonoids occur predominantly in the leaves. In contrast, the concentration flavonoids is low -<1 mg/kg fresh weight- in roots or tubercules; in some cases these compounds may accumulate in the underground parts of certain plants such as onions and radishes. There could be not found any vegetables free from flavonoids.

From the results of HPLC analysis basically became clear that fruits frequently consumed in Hungary did not contain kaempferol and apigenin at all (Table 2). None of five measured flavonoids was detected in any varieties of grapes (Big-grained, Othello), green gooseberry, peach, quince-pear, grapefruit, orange, tangerine and poppy-seed, in oily nuts such as almond, pistachio, kasewnut, groundnut, hazel-nut and coco-nut, and in dried fruits such as raisin, date, fig, and prunes.

Stone fruits have low level of flavonoids except for walnut which is not a really fruit but an oily crop. Only quercetin could be detected in plums and apricot at a concentration range of 11-23 mg/kg. Extremely high level of myricetin was found in walnut (4565 mg/kg), but other flavonoids were not present. Berry fruits seem to be very rich sources of flavonoids. Quercetin was found in sweet and sour cherry (8.9 and 29 mg/kg), in gooseberry (9.1 mg/kg), in strawberry (9.7 mg/kg), in blackberry (14.5 mg/kg), in mulberry (24.7 mg/kg) and blackcurrant (52.8 mg/kg). Extremely high concentration of myricetin was observed in some berries such as redcurrant, mulberry, blackberry, and strawberry (42.9, 452.5, 636, and 993.6 mg/kg, respectively). Fruits similar to apple have luteolin and quercetin at a concentration around 20-30 mg/kg. Two varieties of apple contained quercetin (Gála 30.1 mg/kg, Golden 38.3 mg/kg) and two others luteolin (Golden 27.0 mg/kg, Jonatán 22.5 mg/kg). Pomegranate has also luteolin (18.9 mg/kg). Quercetin was found also in pear (24.7 mg/kg). Water-and muskmelon are very popular fruits in Hungary; they are frequently consumed especially in August. As it can be seen in Table 4 only luteolin was detected in water- and muskmelon and pumpkin (18.4, 25.8 and 16.3 mg/kg, respectively). Among citrus fruits only lemon has flavone luteolin at a concentration of 23.1 mg/kg edible part. From other exotic fruits kiwi contains also luteolin (22.3 mg/kg) and banana has myricetin (22.8 mg/kg).

Estimation of the flavonoid intake in two groups of the Hungarian population

The flavonoid intake was estimated in two groups, first included more than 500 schoolboys and girls aged 12-15 years, and the second group was about 200 healthy adults aged 25-60 years. The average dietary flavonoid intake of the children and the adults was 19.5±26.6 and 18.8±28.9 mg/day, respectively (Table 3). There was no difference between the two groups. At the same time the flavonoid intake showed high differences among the subjects. The consumption of flavonoids in the children's group was from 0 to 179 mg/day, and in the adultsȩ group from 0.5 to 310 mg/day. Opposite of the literary data where the quercetin was said to be the most frequently consumed flavonoid, in our study this compound was myricetin. Myricetin represented 44 - 57% of the total flavonoids while quercetin was 28.6 – 36.1%, and the proportion of kaempferol, luteolin and apigenin was lower than ten percent. With the use of the computing system the average dietary intake of the nutrients such as fat, carbohydrate, protein, energy, micro and macro elements, vitamins, fibre, fatty acids, cholesterol etc. was also calculated in the studied groups (results are not shown). These data were compared to those were calculated during the first representative (in 1985-88) and second (in 1992-94) Hungarian nutrition survey (Bíró 1992; Bíró et al. 1996). These studies emphasized that the daily intake of fat, cholesterol and sodium is very high, while the consumption of other important compounds such as fibre, vitamins, microelements was not high enough. The elements of the improper nutritional habit could be observed in present study. Although two groups in our study did not represent exactly the total

Table 3. Flavonoid intake in two groups of the Hungarian population.

Adults	Quercetin	Kaempferol	Myricetin	Luteolin	Apigenin	Total flavonoids
Number of subjects	204	204	204	204	204	204
Average	6.38	1.12	8.31	1.66	0.85	18.80
SD	6.51	1.51	26.35	2.42	0.87	28.90
Minimum	0.28	0	0	0	0	0.50
Maximum	40.41	14.22	298.9	10.65	5.5	309.67
Children						
Number of subjects	521	521	521	521	521	521
Average	5.56	0.67	11.18	1.55	0.57	19.60
SD	5.54	0.97	25.51	2.24	0.71	26.66
Minimum	0	0	0	0	0	0
Maximum	40.25	11.18	175.9	15.89	4.18	179.35

Hungarian population it is supposed that the average daily flavonoid intake of the population is not different from these data. The estimated Hungarian flavonoid intake is lower than the Dutch (23 mg), Danish (28 mg) and Finnish (55 mg) data (Hertog et al. 1995; Justesen et al. 1997; Kumpulainen et al. 1999). In the Seven Countries study, Japan had the highest intake of flavonoids followed by Croatia, whereas the Finnish cohort had lower intake (Hertog et al. 1995). DeVries and co-workers estimated the intake of five flavonoids in 17 different diets. Lowest intake (1 to 9 mg/day) was from South African diet, whereas highest flavonoid intake (75 to 81 mg/day) was from a Scandinavian diet (deVries et al. 1997). The reason of such very different intakes could be found in the difference of the nutritional habits, the composition of the diets, the flavonoid concentration of the plant foods, and others.

Acknowledgment

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SYMPOSIUM

Effect of age and magnesium supply on the free radical and anti-oxidant content of plants+

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ABSTRACT The scientific literature signs that the shortage of magnesium may increase the free radical content in the animal and human tissues, but nothing can be kown about a similar effect in the plants. This is why we decided to research the plant tissues to know how the radical content and the concentration of antioxidants which may eliminate the free radicals. We have researched the wheat (*Triticum aestivum* var. GK Pinka), maize (*Zea mays* var. Furio) and table beet (*Beta vulgaris* var. Rubra). We measured the concentrations in the coleoptyle of wheat and maize and in the roots of table beet. On the basis of our measurements we may conclude that the increase of magnesium concentration in the nutrient solution does decrease the radical content (HO•, LPO) and the activity of antioxidants. It is known, that the production of free radicals does increase as the uncoupling of oxidative phosphorilation is increasing according to its measure. Nevertheless the reduction of concentration of magnesium increases the measure of uncoupling of oxidative phosphorilation and the production of free radicals. On the basis of these effects one can understand why the radical content is lower after the addition magnesium ions into the nutrient solution. The decrease of activity of activity of GSH, catalase and FRAP-value may be understood also: the real cause is the fact that the promoter free radicals needed to the "de novo synthesis" have decreased. The last conclusion may be the next: the magnesium does influence the of free radicals and antioxidants in the plant tissues.
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KEY WORDS

free radicals
anti-oxidant content
magnesium supply
wheat
maize
table beet

Scientific papers declared that the shortage of magnesium in the animal cell and tissue does increase the free radical content of cell. Kramer et al. (1994) reported that radical content in the heart tissue supplied not enough magnesium was two times higher as in the control tissue supplied by enough magnesium. Wiles et al. (1997) demonstrated that the concentration of free radical containing oxygen did decrease (with 10-15%) when the concentration of magnesium decreased in the aortic endothelial cell. Stafford et al. (1993) presented, that the hydroperoxide level in the rat blood increased in case of magnesium shortage.

We could not find similar phenomena and date in plant cell and tissue. That is why we wanted to know the effect of magnesium on radicals and antioxidants.

Materials and Methods

We have researched the seeds of wheat (*Triticum aestivum*, var. GK Pinka) and maize (*Zea mays*, var. Furio) and the root of table (red) beet (*Beta vulgaris*, var. Rubra).

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+In memory of Professor Béla Matkovics

The germination was carried out in a plastic (PE) dish (which was similar to the Petri dish). The grains were placed on a filter paper covering cotton-wool soaked in distilled water or in solution of magnesium sulphate (0.1% and 1% m/v $MgSO_4 \cdot 7H_2O$). The temperature was 22-24°C during the germinating period. On the 6-8-12th days the shoot (of approximately 25 mm long) was separated. The weighted portion of shoot was homogenised with a Potter type homogeniser and during the process the effective cooling did not allow the temperature increase. The solution used for homogenisation was made of phosphate puffer solution and EDTA (pH = 7,6; EDTA = 1 mM) and it was adjusted to the composition of 1 part shoot to 4 part solution (1:4). After the homogenisation the mixture was centrifuged (5 min 10,000 g) and the upper layer was used for measurements.

We determined the free radical contents in shoots (coleoptyl, epicottyl) of different ages as well as the upper and lower half of coleoptyl. In the case of the coleoptyl, the upper half is younger than the lower half, and we wanted to test whether this slight difference in developmental age would case any detectable difference in the concentration of free radicals.

Table 1. Effect of magnesium on germination of wheat.

Measured components in the coleoptyle of wheat	Measured value Control Without Mg	With 0,1% $MgSO_4 \cdot 7H_2O$	Difference compared	Measured value with 1% to control %	Difference compared $MgSO_4 \cdot 7H_2O$ to control %
Protein, mg/g	11,66	12,12	+4	11,96	+3
LPO, nM MDA/mg prot.	6,7	5,5	-18	5,7	-15
OH*, nM MDA/mg prot.	47,4	43,1	-9	41,9	-12
FRAP value, (mM Fe(II)/L)	532	472	-11	372	-30

Table 2. Effect of magnesium on germination of maize.

Measured component in the coleoptyle of maize	Measured value in the control	Measured value at treating with 1% $MgSO_4 \cdot 7H_2O$	Difference compared to the control %
Protein, mg/g	23,25	24,79	+6,6
LPO, nM MDA/mg prot.	3,75	2,08	-45,0
OH^{Σ}, nM MDA/mg prot.	34,50	18,50	-46,0
GSH, mM/mg prot./10^{-2}	5,67	2,55	-55,0
Catalase, E/mg prot./10^{-4}	0,972	0,316	-67,0
FRAP value, (μM Fe(II)/L)	266	200	-25,0

We also investigated the effects of the total mass and total protein content.

The root of table beet was prepared similar to the kitchen process. After cleaning and the separation the outer layer it was grated with a fine kitchen grater. A weighted portion after addition of distilled water to be adjusted the 1:9 mixture was homogenised with the Potter type homogeniser. As the mixtures made of table beet were very violet-coloured we could measure only the magnesium content and the FRAP value.

We used a magnesium solution (1% m/v $MgSO_4 \cdot 7H_2O$) for spraying the leaves of red beet at growing to research the effect of magnesium on the growing. The spaying was repeated two times per day during the researching period (2 weeks) and the quantity was 0.2 l/m^2 leaves.

The protein content was measured according to Lowry et al. (1951).

The hydroxyl free radicals were determined according to the method of Halliwell and Gutteridge. The HO$^{\bullet}$ radicals at low pH react with 2-deoxy-D-ribose and thiobarbituric acid giving a red coloration. Colour intensity was measured at 532 nm.

LPO activity was measured according to Plancer et al. (1966). The determination is based on the LPO-catalysed reaction of malone-dialdehyde (MDA) with thiobarbituric acid (TBA). The product of reaction is coloured and the colour intensity was measured at 532 nm.

It was determined according to Tietze (1969). The reaction of GSH with the Elman-reagent DTNB (5,5-dithio-bis-[2-nitro-] benzoic acid) yielded a yellow coloration measured at 412 nm.

The determination was made at 240 nm according to Beers et al (1952). The concentration of hydrogen peroxide was controlled in 0,05 M phosphate puffer at pH, measuring the absorbency of solution at 240 nm in silica cell. The enzyme activity was given in Bergmeyer (Be) units (decomposition of 1 g H_2O_2/min at 25°C).

The effect of antioxidant was determined according to

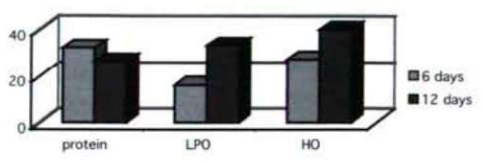


Figure 1. Protein, LPO and HO$^{\bullet}$ content in the 6 and 12 days coleoptyl of maize.

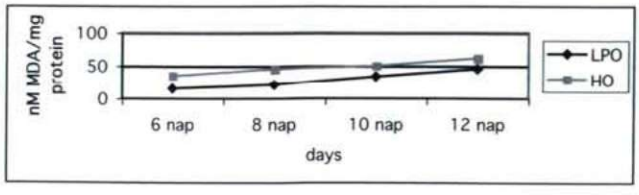


Figure 2. Change of the content of LPO and HO$^{\bullet}$ in the coleoptyl of maize depends on its developmental age.

Table 3. The magnesium content and FRAP value as a function of the species of red beet.

Species of red beet	Mg content, mg/kg			FRAP value		
	Control	Spraying with 1% $MgSO_4 \cdot 7H_2O$	Difference compared to control %	Control	Spraying with 1% $MgSO_4 \cdot 7H_2O$	Difference compared to control
Bordó	1970	2030	+2	330	300	-9
Detroit	2410	2600	+8	312	184	-41
Favorit	1970	2030	+3	343	317	-8
Nero	2040	2160	+3	344	246	-28
Rubin	2130	2270	+7	337	177	-47

Benzie-Strain (1996). The method based on effect of antioxidants to be able to reduce the Fe(III) to Fe(II) in a complex compound (Fe(III)-tripyridine-triazine). The product Fe(II)-compound is blue-coloured and its intensity is measured at 593 nm. After measuring the absorbancy of the blue-coloured solution the concentration can be determined using the calibration curve made by known Fe(II) solutions.

The samples were digested by concentrated HNO_3 solution in microwave oven (0.2 g sample + 5 ml cc HNO_3). After the digestion the solution was filtered and diluted to 50 mL with distilled water. The concentration measurement were made on Jobin Yvon 24 ICP AES instrument.

The spectrophotometric measurements were conducted on the SPECTROMOM 360 and 202 type instruments.

Results

Wheat

The addition of magnesium ion into the germination solution decreased the concentration of hydroxyl free radicals (HO•), and the value of lipid peroxidation (LPO) and FRAP value. In all case the addition of magnesium increased the amount of protein (Table 1).

Maize

All the measured parameters (LPO, HO•, GSH, catalase activity, FRAP value) decreased after the addition of magnesium. (Table 2; Fig. 1). LPO and HO• increased with time (Fig. 2).

Red beet

The fertiliser magnesium (spraying on the leaves) increased the concentration of magnesium and decreased the FRAP value. The effect of magnesium does differ according to the species, but the tendency is similar. (Table 3).

Figure 3 shows the differences of protein, LPO and HO contents between the upper and lower halves as well as the whole coleoptyl. As we can see, the relatively small age difference within the shoot causes a significant deviation in these values, so that one has to take into consideration this fact in collecting shoot samples.

Figure 4 represents the changes of whole protein, LPO, HO, catalase and SOD values in the younger and older epicotyl halves and whole epicotyl of pea plants.

Figure 5 shows the difference when the values are standardised to the usual whole protein content or the total mass.

Discussion

On the basis of our measurements we may conclude that the increase of magnesium concentration in the nutrient solution does decrease the radical content (HO•, LPO) and the activity of antioxidants. It is known, that the production of free radicals does increase as the uncoupling of oxidative phosphorilation is increasing according to its measure. Nevertheless the reduction of concentration of magnesium increases the measure of uncoupling of oxidative phospho-rilation (Vitale et al. 1957) and the production of free radicals. On the basis of these effects one can understand why the radical content is lower after the addition magnesium ions into the nutrient solution.

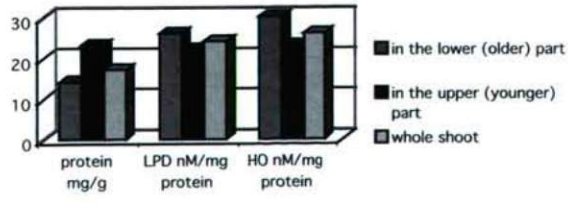
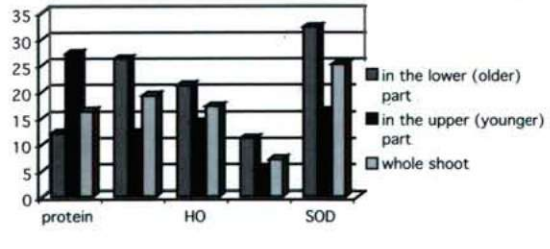


Figure 3. Protein, LPO and HO• content in the 10 days coleoptyl of maize.



Fiure 4. Protein, LPO, HO•, catalase and SOD content in the coleoptyl (epicotyl) of pea.

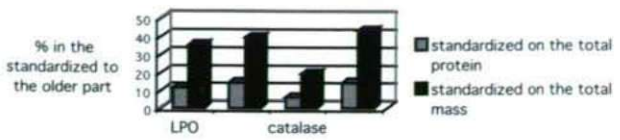


Figure 5. Change of the % of LPO, HO•, catalase and SOD values depend on the age of coleoptyl, standardized to the whole protein content or total mass.

As we can see, the activity of the oxygen free radicals and the anti-oxidants depend on both the age of the whole shoot (coleoptyl and epicotyl) and the slight difference in developmental age between the different parts of the same shoot. The basis of standardisation (protein content or total mass) can cause great differences in the free radical and anti-oxidant values. If standardised to the protein content, the free radical and anti-oxidant values are lower in younger specimens and grow with age. It can be explained with the protein content being higher at younger age than at the older one. When the values are standardised with respect to the total mass, the correlation with age in the opposite. In our opinion, standardisation to the total mass can better reflect reality in certain cases.

The decrease of activity of GSH, catalase and FRAP-value may be understood also: the real cause is the fact that the promoter free radicals needed to the "de novo synthesis" (Vanacker 1998) have decreased.

The last conclusion may be the next: the magnesium does influence the activity of free radicals and antioxidants in the plant tissues, as it was described in case of animal tissue and cell.

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SYMPOSIUM

Further evidence of altered redox status of hyperbilirubinaemic patients: role of bilirubin in Gilbert syndrome+

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ABSTRACT Bilirubin is regarded as the most powerful endogenous antioxidant substance. It exhibits immunmodulator, inhibitor activities on kinases, yet it is clear that it can be potentially cytotoxic. Gilbert syndrome is characterised by hereditary, chronic, mild unconjugated hyperbilirubinaemia. 12 Gilbert syndrome patients and 15 healthy controls were investigated with special regard to reduction-oxidation status and free radical-antioxidant balance. Sera free SH-group concentration, H-donating ability, reducing power were measured spectrophotometric methods. Total scavenger capacity, describing free radical-antioxidant balance, was determined by a newly developed chemiluminometric method in sera, plasma and erythrocytes. Patients with Gilbert syndrome showed a significant increase of non-enzymatic antioxidant capacity. Elevated free SH-group concentration, H-donating ability and reducing power were found in mild hyperbilirubinaemia compared with control group patients. On the other hand no significant differences were detected regarding free radical-antioxidant balance in sera, plasma and erythrocytes between the groups. On the basis of these results it can be supposed that elevated bilirubin concentration, via indirect or compensatory way, strengthens non-enzymatic antioxidant capacity, without changes in antioxidant-free radical balance. That is why further investigations are needed to clarify the consequences of elevated bilirubin concentration on cell redox homeostasis
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KEY WORDS

Gilbert syndrome
redox status
free radical-antioxidant balance
total scavenger capacity

Gilbert syndrome is a chronic non-hemolytic unconjugated hyperbilirubinaemia, occuring in the absence of liver disease or overt haemolysis and characterised by episodes of mild intermittent jaundice. It is the most common inherited disorder of hepatic bilirubin metabolism, occuring of 2-12 percent of the general population. Gilbert syndrome is caused by a reduction in the activity of hepatic bilirubin UDP-glucuronosyl-transferase (UGT1A1) to about 30 percent of normal. The reduction of this gene activity has been shown due to a polymorphism in the promoter region of the UGT1A1 gene, the presence of superfluous thyamin adenine repeats reduces the efficiency of transcription of this gene. The rarer, more severe and dominantly inherited forms identified to date are heterozygosity for a nonsense mutations in the coding region of the UGT1 gene (Bosma et al. 1995; Monaghan et al. 1996; Beutler et al. 1998).

UGT1A1 polymorphism is suggested that may provide a flexible polymorphism that maintain bilirubin levels in a range high enough to protect against oxidative stress, but not so high as to cause a high incidence of kernicterus (Beutler et al. 1998). Bilirubin exhibits intriguing biological activities as an antioxidant, an antimutagen and an anti-complement agent. Some authors suggest that neonatal hyperbilirubinaemia could be a transitional antioxidative mech-anism in the circulation of human neonates (Marilena 1997). Antioxidant property of bilirubin is seemed to be responsible for the reduced risk of coronary artery disease in Gilbert syndrome (Vitek et al. 2002).

Materials and Methods

We studied the redox status and free radical-antioxidant balance in 12 Caucasians patients with Gilbert syndrome, ranging from 18 to 52 years (mean±SEM: 27.08±2.73 years; 11 male, 1 female), in whom the syndrome was clinically diagnosed based on a consistent mildly raised non-fasting serum total bilirubin concentration (range: 25-59 μmol/l) and on the absence of structural liver disease proved by utrasonography. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) values were normal (range ALT: 11-40 U/l; AST: 15-27 U/l). Hemolysis was excluded on the basis of normal hemoglobin value and reticulocyte counts. 16 Caucasians healthy subjects were examined (age:

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Table 1. Routine laboratory parameters in Gilbert syndrome and control patients.

	Total bilirubin concentration (μmol/l)	AST (U/l)	ALT (U/l)	GGT (U/l)	ALP (U/l)
Healthy controls	8.50±0.87	17.00±1.35	15.75±2.21	18.00±5.30	157.53±39.43
Patients with Gilbert syndrome	36.67±2.37	23.17±1.14	28.33±4.50	21.83±3.27	194.83±18.11

27.42±2.35 years; 8 male, 4 female) with no known history of jaundice and with normal serum total bilirubin concentration. (In our laboratory the upper limit of normal for serum total bilirubin concentration is 25 μmol/l. Normal values for AST: 10.00-37.00 U/l; ALT: 5.00-40.00 U/l; GGT: 7.00-50.00 U/l; ALP: 98.00-279.00 U/l)

The study was approved by the Regional Committee of Science and Research Ethics, Semmelweis University (permission number: TUKEB 186/1998). Informed consent was obtained from all subjects. Authors ensure that their work complies with the Declaration of Helsinki (1964).

Plasma and erythrocytes were separated with routine methods and the haemoglobin contents of samples were adjusted to 10 g/l for measurements for Haemisol reagent. The reducing power of the samples were determined at 700 nm according to the method of Oyaizu (1986), based on the chemical reaction Fe (III) → Fe(II). Increased absorbance indicated greater reducing power, which was expressed as the ascorbic acid equivalent (mmol/leqAS). The hydrogen-donating ability of the samples was estimated in the presence of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical at 517 nm on the basis of the method of Hatano et al. (1988). DPPH stable radical was found to oxidise cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds and aromatic amines. Plasma free SH-group concentrations were measured by the Sedlak and Lindsay (1968) method. The total scavenger capacity (TSC) was determined in plasma, sera and erythrocytes by a chemiluminescence assay with Berthold Lumat 9501 manual luminometer, in H_2O_2/OH^--luminol-microperoxidase system to assess the antioxidant capacity and free radicalantioxidant balance. This luminometer is designed to determine single photons of light emitted in the course of a chemical reaction between luminol and free radicals. Intensity of the standard light of H_2O_2/OH^--luminol-microperoxidase system changes depending on the antioxidant-free radical balance in the biological sample and expressed as the percentage of the standard light (RLU%= relative Light Unit%). Higher chemiluminometric intensity indicates lower total scavenger capacity (Blázovics et al. 1999).

1,1-diphenyl-2-picrylhydrazyl, 5,5'-dithiobis-2-nitrobenzoic acid, luminol, microperoxidase were obtained from Sigma (St. Louis, USA). Haemsiol reagent was bought from Human Oltóanyag (Gödöllő, Hungary). All other reagents were purchased from Reanal (Budapest, Hungary).

One-way ANOVA statistical analysis was used to evaluate the significance between patient groups. Data are expressed as mean±SEM. $P<0.05$ was considered to indicate statistical significance.

Results

Total bilirubin concentration in sera showed a significant increase in patients with Gilbert syndrome compared to control group (36.67±2.37 vs. 8.50±0.87 μmol/l). Liver enzymes activities were slightly elevated in hyperbilirubinemic patients, however they did not exceed normal values (Table 1). Significantly elevated H-donating ability (50.68±0.97 vs. 46.11±1.10%), reducing power (1.599±0.004 vs. 1.322±0.088 nmol/leqAS) and free SH-group concentration (0.35±0.009 vs. 0.43±0.013 mmol/l) could be observed in Gilbert syndrome compared to that of control subjects (Table 2). On the other hand no significant differences were observed regarding total scavenger capacity of plasma, sera as well as erythrocytes between healthy controls and Gilbert syndrome patients (Table 3). Bilirubin concentration showed significant correlation only with reducing power (R^2=0.4080, P=0.0253). No significant relation was found in connection with free SH-group concentration (R^2=0.0012, P=0.9142), H-donating ability (R^2=0.2509, P=0.0971) and total scavenger capacity (R^2=0.0655, P=0.4217).

Table 2. Non-enzymatic antioxidant capacity of sera.

	H-donating ability (%)	Reducing power property (nmol/leqAS)	Free SH-group concentration (mmol/l)
Healthy controls	46.11±1.10	264.43±17.59	0.35±0.009
Patients with Gilbert syndrome	50.68±0.97*	319.90±8.08*	0.43±0.013*

*sign. (P<0.05) vs. control group

Table 3. Total scavenger capacity of sera, plasma and erythrocytes.

	Sera (RLU%)	Plasma (RLU%)	Erythrocytes (RLU%)
Healthy controls	20.41±1.61	1.42±0.16	54.11±4.63
Patients with Gilbert syndrome	17.59±1.94	1.44±0.24	55.94±1.76

Discussion

Bilirubin (conjugated/non-bound, non-conjugated/albuminbound) is regarded as a member of the antioxidant family, even though it is known to have toxic effects at high concentrations. Bilirubin could be an important cytoprotector for tissues which are less equipped for antioxidant defence like myocardium and nervous tissues (Marilena 1997). Along with ascorbate and urate, this bile pigment is a very important antioxidant in plasma, can act synergistically with vitamin E in protecting lipid membranes from peroxidation initiated within the lipid phase (Stocker et al.1990; Marilena 1997).

Bilirubin is a potent scavenger of singlet oxygen and peroxyl radicals with high efficiency by initial donation of a hydrogen atom of the tetrapyrrole ring (Stocker et al. 1990; Dudnik and Khrapova 1998; Minetti et al. 1998; Yesilkaya et al. 1998). Bilirubin does react with superoxide anion to some extent and serves as a substrate for peroxidases in the presence of hydrogen peroxide or organic hydroperoxides (Stocker et al. 1987). The superoxide scavenger activity similar to that of serum albumin, higher than the vitamin E analog Trolox and lower than ascorbic acid (Marilena 1997). Bilirubin participates as a scavenger of secondary oxidants formed in the oxidative process (Minetti et al. 1998). Addition of bilirubin to erythrocytes incubated with cumeneOOH (shows a similar effect to the other organic hydroperoxides and produces alkoxyl and peroxyl radicals) induced an inhibition in lipid peroxidation processes, preserving SOD activity, increasing catalase and glucose-6-phosphate dehydrogenase activity and reduced glutathione concentration (Yesilkaya et al. 1998).

Bilirubin also possess antimutagenic properties and conjugated bilirubin shows an inhibitory effect on complement dependent reactions in vitro, blocking complement cascade, especially at the C1 step (Marilena et al. 1998).

It is clear that bilirubin can be toxic to cells at higher concentrations (>30 mg/dl; Mireles et al. 1999). Bilirubin caused cytotoxicity is amplified by TNF-alfa and endotoxin. These results provide a supportive evidence that sepsis would increase the risk of tissue damage by bilirubin, the sepsis may enhance the risk of kerinicterus (Ngai and Yeung 1999). It has been recently reported that bilirubin forms a complex with Cu(II), which results in the reduction of Cu(II) to Cu(I) and this redox cycle gives rise to the formation of reactive oxygen species, particularly hydroxyl radical causing DNA breakage (Asad et al. 1999). It was deduced that bilirubin free radical initiates and promotes the pigment gallstone formation. Bilirubin free radicals were detected by electron spin resonance. The main target of bilirubin free radical is the cell membrane and membrane bound protein (Blázovics et al. 1997; Liu and Hu 2002).

Bilirubin is also a potent immunomodulator: inhibits responses of human lymphocytes, including phytohemagglutinin- induced proliferation, interleukin-2 production and antiobody dependent and independent cell-mediated cytotoxicity (Haga et al. 1996, Maines et al. 1999). This observation may explain the increased susceptibility to infection observed in hyperbilirubinemic patients (Haga et al. 1996). Bilirubin can inhibit protein kinases (cAMP-dependent, cGMP-dependent, Ca^{2+}-calmodulin-dependent Ca^{2+}-phospho-lipid dependent) by a non-competative mechanism, mod-ulating the protein phosphorilation in cellular regulation contributed to its neurotoxicity (Hansen et al. 1996). This kinases initiate and regulate various signal transduction processes including those involved in cell proliferation (Maines et al. 1999).

Earlier studies presumed that elevated bilirubin concentration in Gilbert syndrome serve as an increased antioxidant capacity (Vitek et al. 2002). The object of this study was to investigate the redox status, the free radical antioxidant balance in Gilbert syndrome patients by newly applicated spectrophotometric and newly developed luminometric methods. The mild hyperbilirubinaemia caused elevated SH-group concentration, H-donating ability and reducing power in sera. There were no significant correlations between bilirubin concentration and free SH group concentration as well as H-donating ability. On the other hand strong relation was found between bilirubinaemia and reducing power. On the basis of literature we give the following explanations for our results. Bilirubin may reduce – in consequence its antioxidant property – the utilisation of the free SH group and other antioxidant compounds and molecules, and therefore reinforce antioxidant capacity of tissues. Then again, strengthened antioxidant capacity caused by the mild hyperbilirubinaemia may be in connection with a compensatory mechanism against toxic bilirubin.

On the other hand a shift of free radical–antioxidant balance was not proved by our chemiluminometric mesaurements. We did not detect any significant differences between Gilbert syndrome and healthy control patients in sera, plasma and erythrocytes and tendency was not observed as well. On the basis of these results it can be supposed that elevated bilirubin concentration, via indirect or compensatory way, strengthens non-enzymatic antioxidant capacity, without changes in antioxidant-free radical balance. Further studies

are needed to investigate the consequences of elevated bilirubin concentration in cell redox homeostasis, on the ground its free SH-group concentration elevating, H-donating and reducing power.

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SYMPOSIUM

Genetics of glutathione peroxidase*

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ABSTRACT Glutathione peroxidase is a selenoenzyme responsible for elimination of reactive oxygen species. Since its first identification in 1957 five isoforms have been typified.Several environmental factors, like feed composition, trace element status and vitamin intake, are known to affect the activity of this enzyme, but there are some publications suggesting significant role of genetics, as well. There are some preliminary population level studies on genetic regulation of the enzyme. More recently its molecular genetics was studied primarily. As a result several regulatory elements were identified both on transcriptional and on translational level, but still the full mechanism is not known. Principals of the correlation between glutathione peroxidase activity and production traits is also unclear. This paper is a review of the studies of the authors on this correlation in different livestock species.
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KEY WORDS

glutathione peroxidase
genetics
chicken
mouse
rabbit
inheritance

Glutathione peroxidase (EC. 1. 11. 1. 9) is an important part of the antioxidant defence system. Today five isoforms are known, therefore it is called more like an enzyme family than a single enzyme. They are present in almost every cell of animals, but the tissue distribution of the isoforms shows high variation.

There are several factors abrogating the activity of the enzyme. Some of these are internal, individual factors, resulting in significant variation in the enzyme activity of different organs, age groups and sex. Endocrine regulation can also control enzyme activity.

However, environmental factors have also definite effect on enzyme action. Nutrition is one of the most essential factors as fat content and fatty acid composition of feed, or trace element intake as well as vitamin status of the animal play crucial role in normal enzyme activity. Seasonal changes has also some effect on GSHPx activity as circannual changes have been reported in the literature (Erdélyi et al. 1999).

Recent publications also suggest that genetic regulation has important role in the control of enzyme action. Most of these studies are done on the population level. In some preliminary experiments glutathione peroxidase (GSHPx) activity was found to correlate with several production traits. In Finn sheep, for instance, negative correlation was shown between enzyme activity and body weight, weight gain (Atroshi and Sankari 1981). Similar results were obtained with chicken (Lavronga and Combs 1982), pig (Lingaas et al. 1991) and rabbit (Mézes et al. 1994). Some phenotypic variance of the enzyme activity and its correlation with genetic muscle dystrophy was reported in chicken (Hull and Scott 1976) and in mice (Bell and Draper 1976).

In this paper authors wish to present an overview of their experiments on population level correlations between GSHPx activity and production traits in some livestock species.

Materials and Methods

Animals and samples

Experiment 1

Pure-bred Landes, Pure-bred Hungarian White, Landes x Hungarian White and Hungarian White x Landes goose stocks were involved in the study. Liver samples (n=12/lines) were taken at 2nd and 70th days of age.

Experiment 2

Compact and control mice as well as their two different crossbred lines (in one mothers were compact in the other the father was compact genotype)were involved in the experiment. Liver samples were taken from mice at 14, 28, 42, 56 and 90 days of age.

Experiment 3

Liver samples were collected from day old chickens of six genotypes. The studied six genotypes were as follows: Plymouth Rock White (PRW), Naked Neck Plymouth (NNP), Naked Neck New Hampshire (NNNH), Hungarian

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*In memory of Professor Béla Matkovics

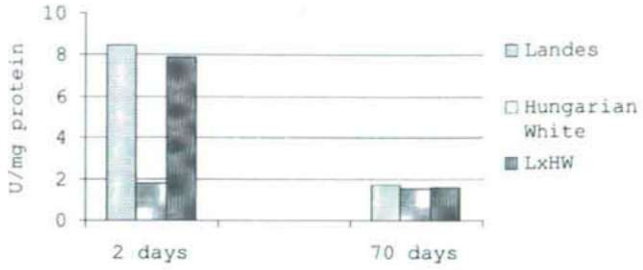

Figure 1. Glutathione peroxidase in goose liver in different age in different genotypes.

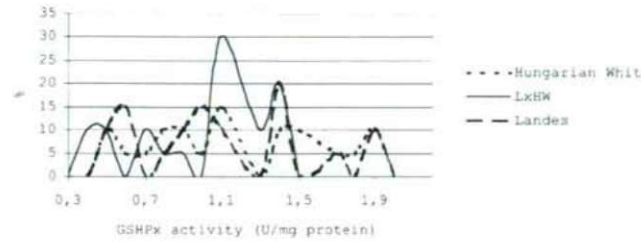

Figure 3. Distribution of GSHPx activity in the population of the three genotypes at 70 days of age.

Speckled (HS), Transylvanian Naked eck White (TNNW) and Hungarian White (HW).

All the experiments were prepared according to the guidelines of Hungarian Law for protection of animal rights.

Sample preparation for biochemical analysis

Liver samples were homogenised in 1:9 physiological saline solution. Homogenates were centrifuged and 10.000 g supernatant was used for further analysis.

Fat content of goose liver samples were removed after a preliminary centrifugation at 500 g for 5 min previous to the high speed centrifugation.

Glutathione peroxidase activity was measured with an end-point direct assay according to (Matkovics et al. 1988). Activity data were referred to protein concentration of the samples. To measure protein concentration Folin phenol reagent was used (Lowry et al. 1951).

Statistical analyses

The differences among different genotypes were calculated using analysis of variance and regression analysis (Snedecor and Cochran 1976) and the levels of significance were assessed with Duncan's test.

Results

Experiment 1

Hungarian White genotype has significantly ($P<0.001$) lower GSHPx activity in the liver of two days old gooslings, than pure bred Landes or the cross bred genotypes (Table 1). While no significant differences were found among the three latter groups.

The difference in the early postnatal age disappeares by 70 days of age (Fig. 1).

Studying the distribution of enzyme activity in the population, distribution peaks were well separated in the three genotypes and Hungarian White was quite far from the two other groups concerning enzyme activity distribution (Fig. 2).

These differences are faded away at 70 days of age as distribution peaks of the different genotypes more or less overlap each other (Fig. 3).

Experiment 2

There was significant difference between the control and compact group at 28 days of age ($P<0.001$) in the males, while enzyme activity of the crossbred lines were similar. It

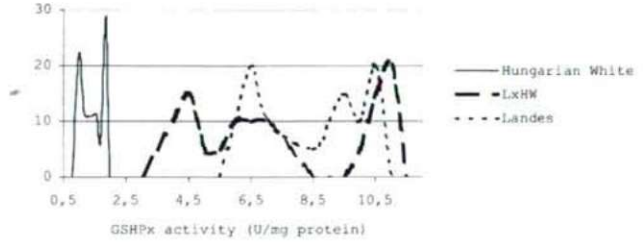

Figure 2. Distribution of GSHPx activity in the population of the three genotypes at 2 days of age.

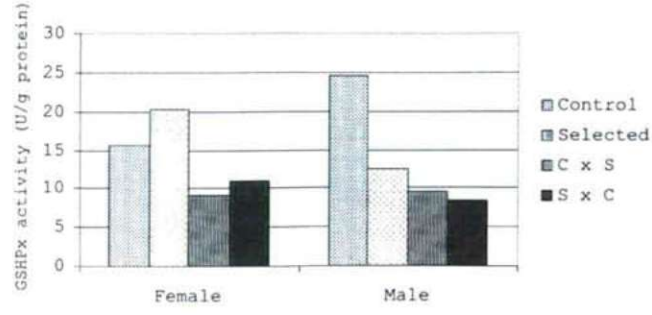

Figure 4. Glutathione peroxidase activity in the two sexes of mice at 28 days of age.

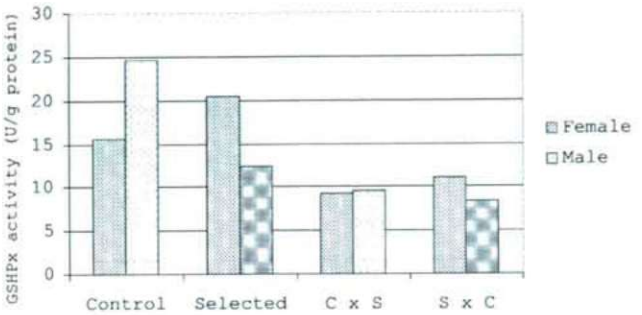

Figure 5. Glutathione peroxidase activity in the four lines of murine at 28 days of age.

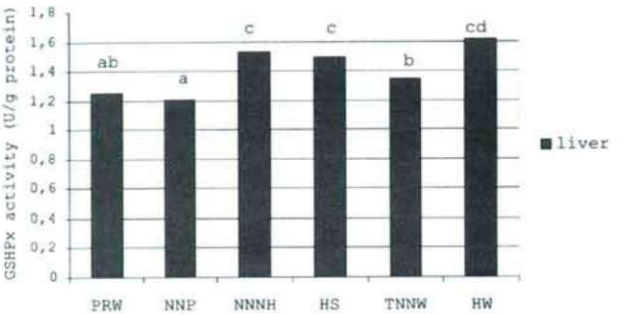

Figure 6. Glutathione peroxidase activity in the liver of different chicken genotypes (different letters mean significant difference at $P<0.05$).

is also important that these latter values were closer to that of the compact mice than the control (Fig. 4). There was no significant difference among the enzyme activity of the four lines in the females. While there were no significant changes at any other sampling period.

In the control line males had higher GSHPx activity than females, while in the compact line enzyme activity of the two sexes were the opposite. Comparing the data of the two crossbred lines controversial results were obtained in the two sexes. Glutathione peroxidase activity of males in the line having compact father was lower as compared to the values of males in the other crossbred line, while the same data for females were just the opposite. In the line having control father, glutathione peroxidase activity was almost the same in the two sexes. In the line having compact father, glutathione peroxidase activity of females were higher than that of the males (Fig. 5).

Table 1. GSHPx activity in the liver homogenates of two days old gooslings of different genotypes (different letters mean significant difference at $P<0.001$)

Genotype	GSHPx activity (U/mg 10.000g supernatant protein)
Hungarian White	1.47 ±0.45 a
Landes	8.72 ±1.85 b
Landes x Hungarian White	7.86 ±3.44 b
Hungarian White x Landes	10.42 ±6.33 b

Experiment 3

Concerning phenotypic variation of glutathione peroxidase activity in liver homogenates of the different genotypes NNP was found to have significantly lower enzyme activity than all the other groups except PRW phenotype. Highest enzyme activity was measured in the individuals of Hungarian White type (Fig. 6).

According to correlation analysis negative correlation was found between glutathione peroxidase activity and body weight. Strength of correlation increases with age from 4 weeks to sexual maturation. Also negative correlation was shown between enzyme activity and weight gain among each age group, except age of highest egg production, while positive correlation was found between liver GSHPx activity and egg production (Table 2).

Discussion

Experiment 1

Glutathione peroxidase has significantly higher activity at 2 days of age in the liver of Landes type geese. Cross bred variety has GSHPx activity more like Landes than the other parent variety.

Fatty liver production of Landes breed has very good genetic inheritance. Glutathione redox system, including glutathione peroxidase is thought to have importance in fatty liver production.

Therefore, our results suggest that glutathione peroxidase activity of the liver of 2 days old gooslings might be a

Table 2. Correlation between liver glutathione peroxidase activity and performance traits.

	WG 0-4	WG 4-8	WG 8-12	WG 12-SM	BW 4.w.	BW 8. w	BW 12. w	BW SM	BWHEP	Egg prod.
Liver GSHPx act. correlation	-0.49	-0.71	-0.31	-0.60	-0.49	-0.6	-0.77	-0.83*	-0.77	0.54

possible marker for prediction of lipogenetic activity of liver, hence it can be used as selection marker for early selection for liver production efficiency.

Differences of GSHPx activity among different varieties at early postnatal life were disappeared in adult animals, which shows the impotance of age as a criteria to give normal range in glutathione peroxidase activity in avian species.

Experiment 2

Phenotypic difference between control and compact mice is at least in part a result of biochemical processes related to lipid metabolism. According to the results, glutathione peroxidase might be a potential selection marker for compact characteristics, but the two sexes should be concerned separately as enzyme activity varied between females and males.The best study period is between 4-8 weeks of age in mice considering the largest variation of glutathione peroxidase activity at this time.

As compact caracteristics is a preferable one in livestock animals, measuring enzyme activity could be a good indicator of meat production performance even if compact phenotype is not present in a population.

Experiment 3

There is relatively high differences between genotypes in glutathione peroxidase activity and these correlate well with some of the performance traits even in one day old chicken. This provides a potential early selection marker for production traits, but to put this into practice further experiments are required.

Altogether, according to the results of experiments introduced above, glutathione peroxidase activity is a real potential selection marker as it shows correlation to special production traits, and changes in different genotypes. To determine the ways of its use further, large scale selection experiments should be done.

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SYMPOSIUM

Some effects of lead contamination on liver and gallbladder bile+

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ABSTRACT Lead can cause liver damage in which free radical reactions are involved. In order to study the effect of lead on liver and gallbladder, we examined human gallbladder bile after cholecystectomy, as well as the liver and bile of broiler chickens fed with basal diet and contaminated with 400 and 600 mg/kg lead ($Pb(CH_3COO)_2$. Concentrations of lead in human bile were determined with inductively coupled plasma optical emission spectrometer (ICP-OES). Diene-conjugate content as well as thiobarbituric acid reactive compounds in bile were determined by spectrophotometry. In the case of lead poisoning good correlation was observed between the lipid peroxidation parameters of bile and the picture of necrotised tissue observed in a histological study.High lead contamination caused mainly liver damage while cholecystitis was induced by the low concentration of lead, probably, by changing the normal biliary processes.

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KEY WORDS

lead
gallstone
bile
lipid peroxidation

Lead poisoning generally results from well-known occupational exposure *e.g.* crystal ware, glazed pottery, however, in some instances it may arise from unexpected sources *e.g.* home made wine. Lead may cause colicky abdominal pain, weight loss and elevation in liver function tests, although in 29% of these patients no complaints have been recorded (Janin et al. 1985).

In previous studies some difference was found in the metal concentration of bile between gallstone patients and control subjects. Significantly lower concentration of lead was found in bile samples from gallstone patients than from controls, although the excretion of lead was supposedly the same (Calderon et al. 2000).

The purpose of the study was to determine the effects of lead on the hepatobiliary system, especially on the liver and on the gallbladder.

Materials and Methods

Humans

Between January 1998 and December 1999, 40 patients with 60.51±14.34 years of age, and men/women ratio of 8/32 were involved in the experiments. A questionnaire was filled out to determine occupational or home lead intoxication. Gallstones and bile samples were obtained. The bile was taken by needle aspiration and the total bile volume of the gallbladder was determined. The gallbladder was prepared by routine histological methods and the degree of inflammation was determined. The fasting gallbladder volume was calculated from the sucked bile. The research was approved by the ethical committee of the institution (No.59/1996) and followed institutional guidelines for the care and use of laboratory animals (No.45/1999).

The concentration of lead in human bile and in gallstones was determined by ICP-OES. Bile fluid samples (2.0 g) and gallstones were digested with a mixture of HNO_3 (5 ml) and H_2O_2 (3 ml) in teflon vessels. After digestion the samples were diluted to 25 ml with deionised water (Szentmihályi et al. 2000).

Animals

Broiler chickens were fed with basal diet and treated with 400 and 600 mg/kg lead ($Pb(CH_3COO)_2$) ad libitum from 1 week to 6 weeks of age. At the end of the experiments the chickens were decapitated, and the liver and gallbladder bile were removed. The samples were stored at –20°C during the measurements (Blázovics et al. 2001).

The volume of liver homogenates was 0.05 ml (protein content: 10 mg/ml) measured by the method of Lowry

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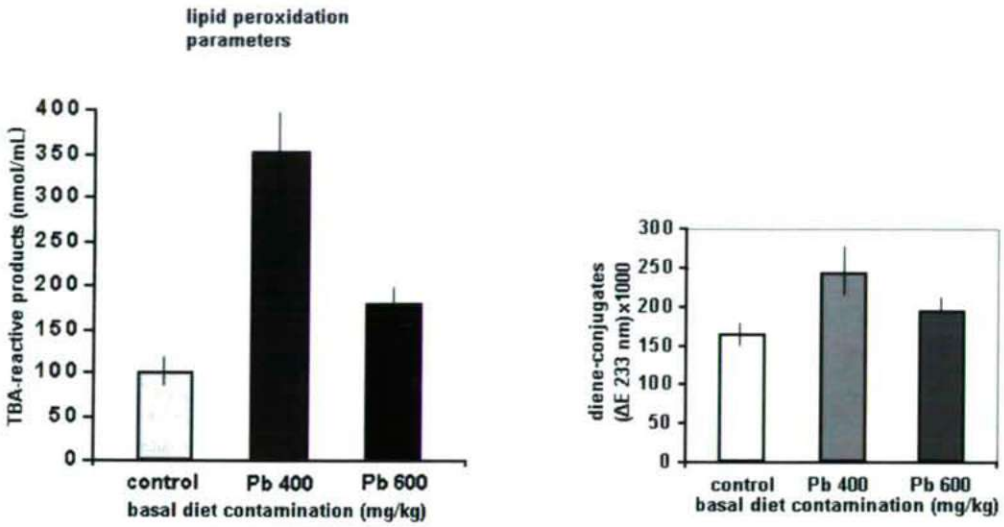

Figure 1. Lipid peroxidation processes in the bile of broiler chicken treated with lead. The decreased concentrations of thiobarbituric acid reactive compounds and diene conjugate content of bile in higher dose of lead reveal aggregation processes in the gallbladder bile.

(1951). Thiobarbituric acid reactive compounds of the bile were measured by the method of Pyles (1993) and the diene conjugate content of the bile was determined at 233 nm by spectrophotometry after fractionated separation of isooctane, then desiccated with Na_2SO_4 (AOAC 1993). Routine histopathological examinations were performed. All reagents were purchased from Reanal (Hungary). Mean and standard deviations were calculated from the results and significance was determined by Student's *t* test.

Results

Pb was detected in 9 bile samples from the above 40 patients with gallstones (23%). One patient worked with ceramic dye, one performed renovating work at home and the others were not exposed to Pb. Average lead concentration was 0.2±0.21 mg/kg. On an average, the Pb content of bile was 0.228±0.124 mg/kg, measured from 6 samples taken from 40 patients. Human gallstone Pb content was 20.55±48.69 mg/kg observed in 3 samples in very high concentration. Elevated Pb concentration in gallbladder bile and stone was detected only in samples of one patient. In the other cases, the Pb content of bile fluid and gallstone did not show parallel changes. The volume of gallbladder with lead content was significantly lower than observed for gallbladder without lead content (7.8±2.3 ml/21±12.4 ml), but histological preparation did not reveal any thickening of the gallbladder wall. The gallbladder volume with muddy bile content was always lower and could not be entirely sucked out, but the remaining volume was always lower than 10%.

In order to prove that Pb may cause lipid peroxidation injuries in the liver and gallbladder, we studied the effect of lead in broiler chicken experiments. When the basal diet of chickens was contaminated with 400 and 600 mg/kg $Pb(CH_3COO)_2$ the diene-conjugate content and concentration of thiobarbituric acid reactive substances were determined in broiler chicken gallbladder bile supernatants (Fig. 1). Similar ratio was observed between the two parameters in heavy metal poisoning. These data point to lipid peroxidation processes in the gallbladder bile. The amount of sludge was significantly higher and the volume of gallbladder was lower in highly poisoned chickens (600 mg/kg), than observed in the control animals. Histological sections confirmed the dose-dependent toxic effect of lead in the liver, treatment with 400 mg/kg lead caused lymphocyte infiltration, and 600 mg/kg lead treatment caused severe periportal inflammatory reaction, which may lead to cirrhosis (Fig. 2).

Discussion

Lead has been shown to be toxic in most of its chemical forms, either inhaled or ingested in water or food at levels humans are exposed to at the workplace as well as in the general environment. Gastrointestinal lead absorption and retention, the major pathway of lead intake, were shown to vary widely depending on the chemical environment of the gastrointestinal lumen, age and iron stores (nutritional status of the subject). Lead does not have a feedback mechanism, which limits absorption. Dietary components, such as, sodium citrate, ascorbic acid, amino acids, vitamin D, proteins, fat and lactose can bind to lead and thus enhance the absorption of lead (DeMichele 1984). When lead is ingested or absorbed in the body, blood Pb level initially rises and then falls within days. After prolonged exposure, lead is

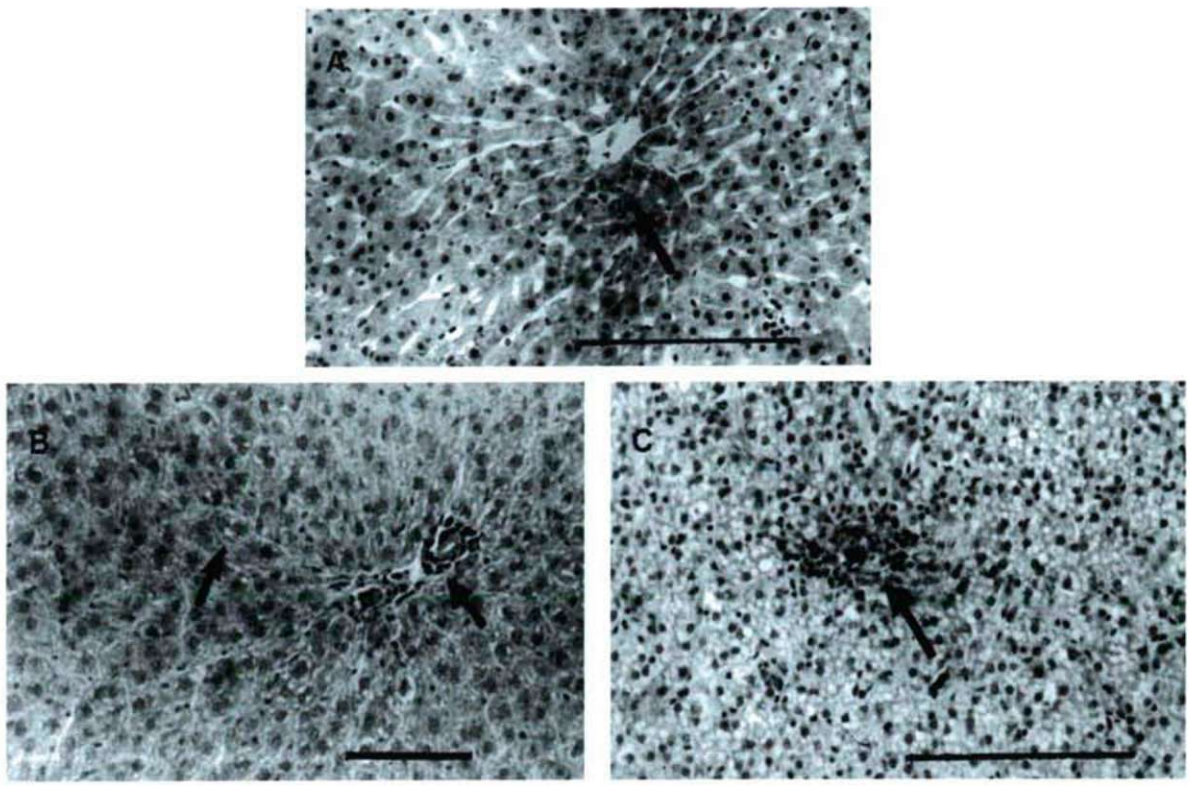

Figure 2. Light microscopic picture of the liver exposed to lead. **A**: control; **B**: 400 mg/kg lead; **C**: 600 mg/kg lead; **A**: central part of a hepatic lobule of a control animal. Bar scale = 100 mm, **B**. and **C**: parts of the hepatic lobules from a led treated chicken. Arrows show micro lipid droplets. Dotted arrows point to the periportal inflammatory reactions.

stored in the skeleton where the time of equilibration with blood and organs takes several years.

The significance of our study is that it draws attention to the fact that the cause of abdominal pain may be lead poisoning as well, which may be induced by changes in the visceral smooth muscle tone secondary to the action of lead on the visceral autonomic nervous system, as well as lead induced alteration in sodium transport in the small intestinal mucosa.

Lead can stimulate intercellular signalling between Kupffer cells and hepatocytes, which are enhanced synergistically in the presence of low lipopolysaccharide levels (Milosevic and Maier 2000) promoting thereby proteolytic activity.

Our results have shown that treatment with higher concentration of lead causes severe periportal inflammation in chicken liver, therefore, it may be assumed that long-term lead exposure might cause liver damage in humans as well.

Good correlation was found between the elevated lipid peroxidation parameters and the decreased total scavenger capacity of the liver exposed to lead (Blázovics et al. 2001).

In general, the different effects of lead in the hepatobiliary system are: catalysis of the peroxidation of unsaturated fatty acids (Yin and Lin 1995), reduction of N-oxide production (Krocova et al. 2000) and the promotion hydroxyl radical formation (Ding et al. 2000).

Each of these affects may promote stone formation. The proliferation effect of lead on smooth muscle cells (Fujiwara et al. 1995) and the rise in the tone of gallbladder may result in smaller gallbladder volume and an increase in wall thickness.

Our results show that higher concentration of lead causes mainly liver damage in which free radicals are involved, but low concentration of lead may disturb the normal biochemical process in the hepatobiliary system and the lead may precipitate into gallstones.

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SYMPOSIUM

Effects of chicory on pancreas status of rats in experimental dislipidemia+

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ABSTRACT The well known medicinal plant, *Cichorium intybus* L. (Asteraceae) contains dietary oligofructose compounds which have beneficial effect on carbohydrate and lipid metabolism, other compounds e.g. polyphenol type derivatives could be responsible for the antioxidant properties reported in several studies before. Male Fischer rats were kept on normal and lipid rich diet supplemented with chicory extract in this study. Our aim was to investigate the influence of this supplementation on pancreas status of rats, especially in dislipidemia. Polyphenols, flavonoids and caffeic acid derivatives were measured in chicory extract by spectrophotometric and chromatographic methods. Antioxidant property of the plant extract was determined *in vitro* and the biological activity of antioxidant compounds of chicory was investigated *in vivo* by a luminometric technique.The effects of bioactive molecules of chicory extract influenced the lipid metabolism and the redox balance of pancreatic tissue of rats in experimental dislipidemia. **Acta Biol Szeged 47(1-4):143-146 (2003)**

KEY WORDS

Cichorium intybus
hyperlipidemy
pancreas
antioxidant status

Cichorium intybus L. (Asteraceae) as an important medicinal herb, has been used in folk medicine for liver disorders, gallstones and for inflammations of the urinary tract since the 17th century. It is well-known from the literature that the main active compounds of chicory are: inulin, fructooligosaccharides, caffeic acid derivatives, flavonoids and polyphe-nols.

Chicory fructooligosaccharides have been investigated in studies on the gastrointestinal system especially because of the inulin and dietary fiber content of this medicinal plant. Some oligosaccharides have functional effects similar to soluble dietary fiber such as enhancement of a healthy gastrointestinal tract, improvement of glucose control, and modulation of the metabolism of triglycerides (Roberfroid 2000; Roberfroid and Slavin 2000).

Dietary supplementation with oligofructose (100g/bwkg) a non-digestible oligomer of beta-D-fructose decreases triacylglycerols and VLDL in serum rats. By measuring the activity of key enzymes (*e.g.*: fatty acid syntase, phosphatidate phosphohydrolase) the authors found that long term feeding with oligofructose protected rats against liver triacylglycerols accumulation induced by fructose (Kok et al. 1996).

Oligofructose significantly alters liver lipid metabolism, resulting over time in a significant reduction in plasma triacylglycerols, phospholipids and cholesterol levels (Fiordaliso et al. 1995).

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The water soluble antioxidant properties of *Cichorium intybus var. silvestre* were investigated and evaluated *in vitro* and *ex vivo* as protective activity against rat liver cell microsome lipid peroxidation (Gazzani et al. 2000).

The aim of our studies was to verify the effect of chicory decoct on lipid metabolism and antioxidant defend system in a short term animal experiment observing the changes in pancreas function through biochemical measurements. We also investigated the radical scavenger property of chicory extract *in vitro* with the help of chemiluminescence measurements.

Potential bioactive constituents (polyphenols, flavonoids and caffeic acid derivatives) of chicory extract were measured by spectrophotometric and cromatographic techniques.

Materials and Methods

In this short term experiment young male Fisher rats (weight: 200 ±25 g) were kept on normal diet (20 animals) and lipid rich diet (20 animals). In the normolipidemic groups, rats were fed with normal chow (CRLT-N, Biopharm Prompt Kft., Hungary). In the hyperlipidemic groups, rats were kept on lipid rich diet (20% sunflower oil, 2.0% cholesterol, 0.5% cholic acid added to the normal chow) ad libitum according to their growth. Ten animals fed with normal diet and ten animals fed with lipid rich diet were treated by gastric tube with the solution of the lyophilised chicory decoction (2 g/ body weight kg) for 10 days.

The blood was collected by cannulation of posterior vena cava in deep Urethane anaesthesia (35 mg /body weight kg).

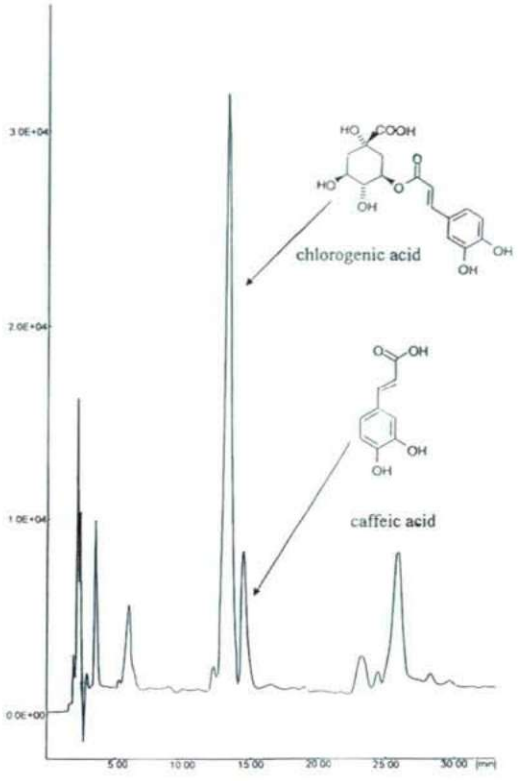
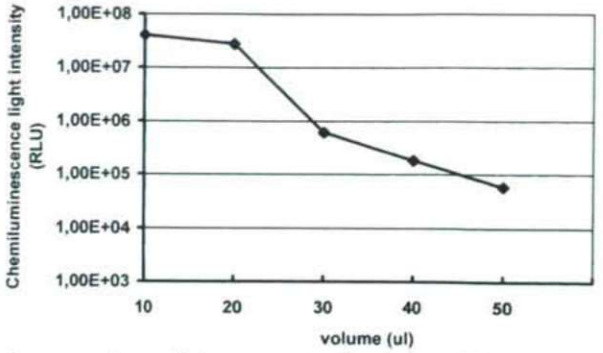


Figure 1. Qualitative evaluation of chicory extract by HPLC.



Each measuring point represents the mean of five parallel data. The C.V. % was below 5% respectively.

Figure 2. Changes of chemiluminescence light intensity in H_2O_2 /°OH microperoxidase system depending on the different volumes of Chicory decoct solution (1g %).

Sera for the biochemical measurements were separated by centrifugation at 2800 RPM for 10 minutes.

The total radical scavenger capacity (TSC) of pancreas tissue homogenates was measured by a chemiluminescence assay in H_2O_2/°OH-microperoxidase-luminol system adapted to Berthold Lumat 9501 instrument (Blázovics et al. 1999). During measurement, the program gave the integrated value of the light reaction. Light emission was initiated by the addition of 0.050 ml of 70 μmol l^{-1} alkaline luminol solution (pH 9.8), 0.30 ml H_2O_2 (1:10,000 dilution) and 0.30 ml of 1 mmol l^{-1} micro-peroxidase solution as catalyst. The sensitivity of the instrument allows detection limit of <0.1 pg of material.

Enzyme activities (alpha-amylase (AMYL), lipase) and lipid parameters (cholesterol (CHOL), triacylglycerols (TG)) from the sera of animals were determined by Hitachi 717 automated chemical analyser using spectrophotometric enzymatic methods. For the measurement of alpha-amylase activity, ethylidien-G_7PNP was used as substrate in method of Lorentz et al. (Lorentz 1998). Lipase activity was measured by turbidimetry with colipase using the method of Lott, on DTN 400 spectrophotometer (Lott 1986). Cholesterol concentration was measured by enzymatic colorimetry (Richmond 1973) and concentration of triacylglycerols was determined by the enzymatic method of Nagele et al. (1984).

Pancreas tissue was homogenized in 0.9% NaCl solution, with a Potter-Elvehjem homogenizer, equalised by dilution to 5 mg% protein concentration. Protein content was measured by Lowry et al. (1951).

Wild growing *Cichorium intybus* L. (Asteraceae) were collected during flowering, identified in the Department of Pharmacognosy, Semmelweis University, where vouchers have been deposited. Diluted alcoholic (40 v %) extract (1:5) was prepared from the whole plant, which was then concentrated and lyophilised.

Plant extract was standardised for the potential bioactive constituents. Total polyphenol content was measured by the method of the Hungarian Pharmacopoeia (Ph.Hg. VII.), results were expressed as pyrogallol. Flavonoid content was determined by the modified method of the German Pharmacopoeia (DAB 10) by spectrophotometry following acidic hydrolysis. Result was expressed as hyperoside.

Table 1. Changes of the main lipid parameter concentrations in sera of rats in the different dietary groups.

Dietary groups	CHOL (mmol/l)	TG (mmol/l)
Normal diet	2,07*±0,15	0,81 ±0,06
Normal diet supplemented with chicory extract	2,11 ±0,12	0,56 ±0,05
Lipid rich diet	10,27* ±1,13	1,23*±0,20
Lipid rich diet supplemented with chicory extract	9,68 ±0,48	0,64* ±0,05

* significantly different ($p<0.05$)

Caffeic acid derivatives were measured by spectroscopic method (325 nm) after purification using caffeic acid for calibration. Results were expressed as caffeic acid.

Qualitative composition of the extract, used in the experiments was characterised by HPLC fingerprint. HPLC separation was performed with an ABL & JASCO system consisiting of PU-980 gradient pump and RHEODYNE 7725 (20 μl) injector. The instrument was equipped with a PU-980 UV-VIS detector in combination with an IBM-PC. A Hypersil ODS (5 μm) reverse phase C-18 column (250 x 4 mm) protected with a precolumn of the same material was used. Two solvent mixtures were employed for elution: Eluent A: AcCN, Eluent B: H_2O : CH_3COOH (40:1). Separation was achieved at ambient temperature with a flow rate 1.0 ml min^{-1}. Gradient elution was used. Data were collected at 325 nm. Peaks were identified with authentic standards by accordance to UV spectra and retention time.

The biochemical reagents were products of Roche Diagnostics, luminol and microperoxidase were purchased from Sigma Chemical (USA). All other reagents were obtained from Reanal (Hungary).

For the statistical analysis the STATISTICA 6.0 software package was used. The values were expressed as mean ±SD, at $p< 0.05$, the difference was considered significant.

Results and Discussion

Numerous experimental studies published in the last decades investigated the possible involvement of reactive oxygen species in acute pancreatitis (Sanfey 1984; Nonaka 1989; Gough 1990; Tsai 1998). Most of them applied measurements for observing of the loss of endogenous antioxidants (ascorbic acid, glutathione) and the increase in markers of oxidative damage (malondialdehyde and 4-hydroxynoneal; Kruse et al. 2001).

Quantitative evaluation of chicory extract shows flavonoids as hyperoside 0.12 ±0.02 g%, polyphenols as pyrogallol 12.98 ±0.06 g%, caffeic acid derivatives as caffeic acid 16.7 ±0.306 g%. Qualitative composition based on HPLC fingerprint showed chlorogenic acid, caffeic acid and the luteolin flavone as characteristic compounds (Fig. 1).These components may be responsible for the *in vitro* antioxidant property of the natural extract shown by Figure 2. The intensity of the emitted light in H_2O_2/'OH-luminol - microperoxidase system was diminished depending on the concentration of chicory decoct (Blázovics et al. 1999).

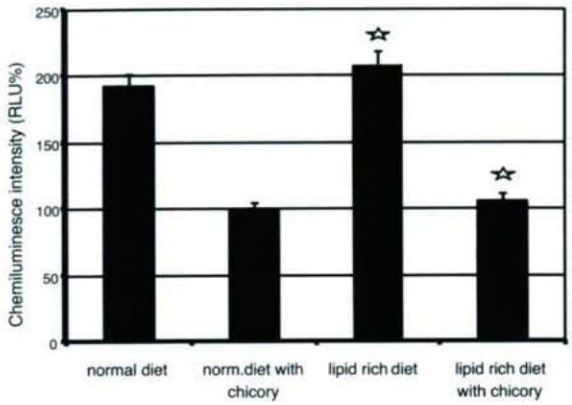


Figure 3. Changes in chemiluminescence intensity (RLU %) in pancreas tissue homogenate of rats in the different dietary groups. Signal * means significant difference ($p<0,05$).

The status of dislipidemia was verified by the increase of cholesterol and triacylglycerols concentrations in the sera of animals fed on lipid rich diet. In the dislipidemic group of animals, which received plant extract supplementation by their lipid rich diet, lower cholesterol and triacylglycerols concentrations were found (Table 1; Blázovics et al. 2000).

In the group of the animals fed on normal diet applying chicory extract supplementation, the activities of pancreas enzymes: alpha-amylase, and lipase in the sera showed slight increase, which were not significant. In the dislipidemic and plant extract treated animal group, lipase level was significantly lower than that of hyperlipidemic group. The alpha-amylase activities were not changed (Table 2).

These chemiluminescence measurement of the samples prepared from pancreas tissue homogenate of rats, as the intensity of the chemiluminescence light emission (RLU%) reduced significantly from 191.9% to 99.3% ($p< 0.05$) in the extract treated group by normal diet, and from 207.0% to 105.0% ($p<0.05$) in the dislipidemic animal group, showing the effect of decoct supplemented diet on pancreas status (Fig. 3).

On the basis of our results beneficial effect was verified on pancreas status in rats, using chicory extract supplementation either by normal or by lipid rich diet. We could reveal the change of the antioxidant status of pancreas tissue

Table 2. Changes of pancreas enzyme activities in sera of rats in the different dietary groups.

Dietary groups	LIPASE (U/l)	AMYL (U/l)
Normal diet	17,5 ±3,12*	6306,5 ±289,7
Normal diet supplemented with chicory extract	12,71 ±2,95	6675,8 ±366,2
Lipid rich diet	29,33 ±8,83*	8603,2 ±847,2
Lipid rich diet supplemented with chicory extract	15,6 ±1,78*	8660,2 ±451,1

*significantly different ($p< 0.05$)

of rats to the effect of chicory extract supplementation, with the help of chemiluminescence method.

Acknowledgment

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SYMPOSIUM

Heme oxygenase 1 (HMOX1) gene expression in hemodialysed uremic patients*

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ABSTRACT The oxidative stress and the released heme molecule of degrading red blood cells during hemodialysis induce the expression of heme oxygenase 1 gene (HMOX1). The goal of our study was to measure the expression of HMOX1 during dialysis and compare it with the common oxidative markers. We established a cRT-PCR (competitive reverse transcriptase PCR) method to measure the mRNA levels. The mRNA levels were calculated to the white blood cell count respectively for each sample. We measured a 1-5-fold increase in the HMOX1 gene expression after the dialysis session, which dropped back to the original value 48 hours after the dialysis. The base level of HMOX1 mRNA of hemodialysed patients before dialysis was in the range of normal controls. The changes in mRNA levels showed significant correlation with the plasma hemoglobin ($r=0.72$, $p<0.001$) and plasma bilirubin ($r=0.71$, $p<0.002$) changes. We found no correlation between the changes of mRNA levels and other biochemical markers (GSH, GSSG). Our results suggest that the induced HMOX1 expression during hemodialysis is primarily due to the undergoing hemolysis. **Acta Biol Szeged 47(1-4):147-151 (2003)**

KEY WORDS

oxidative stress
hemodialysis
heme oxygenase 1
gene expression
cRT-PCR

Oxidative stress is known to play an important role in the etiology of numerous human conditions, including atherosclerosis, cerebral ischemia, and several neurodegenerative, neuromuscular disorders and renal injury. Cellular antioxidants appear to be crucial for the reduction of ox-idative stress and the prevention of associated pathology. Of the known enzymatic antioxidant systems, perhaps the best characterized are superoxide dismutases, catalases, and glutathione peroxidase, which directly metabolize free radical precursors (Coyle and Puttfracken 1993). A group of stress proteins including heme oxygenase 1 are up regulated during oxidative stress and ostensibly act to maintain the structural and functional integrity of damaged proteins, and thus plays cytoprotective role. Heme oxygenases catalyze the rate-limiting step in heme degradation, resulting in the formation of iron, carbon monoxide, and biliverdin, which is subsequently converted to bilirubin by biliverdin reductase.

Heme is a tetrapyrole with a redox active iron center, and functions as a co-factor for various proteins such as hemoglobin, myoglobin, cytochromes, catalases (Balla et al. 1993; Muller-Eberhard and Fraig 1993). In this role heme is essential for many biological process including oxygen transport and energy production. Tissue damage or cell injury can destabilize heme-proteins and result in "free" hem, which in turn can damage cellular components and disrupt cellular function (Balla et al. 1991a, 1991b; Nath et al. 1995, 1998). Heme itself promotes to the oxidative degradation of DNA (Aft and Mueler 1983), proteins (Aft and Mueler 1984) and amplifies the hydrogen peroxide mediated endothelial cell dysfunction (Balla et al. 1993). The elimination of excess "free" heme is done by the heme oxygenases (HOs). At present three functional isoforms of HO are known (HO-1, HO-2 and HO-3). HO-2 and HO-3 are constitutional form while HO-1 is inducible by heme (Alam et al. 2002), and various other physiological and non-physiological stimuli such as hemin (Shibahara et al. 1978), inflammatory cytokines (Rizzardini et al. 1993; Kutty et al. 1994), UV-irradiation, heavy metals and arsenite (Keyse and Tyrrel 1989). The HO-1 obviously plays a cytoprotective role in oxidative stress (Stocker 1990) and heme mediated injury (Nath et al. 1995, 2000, 2001), which is also confirmed by the knockout animal model (Poss and Tonegawa 1997). Furthermore HO-1 has been implicated in many clinically relevant disease states including atherosclerosis, transplanted graft rejection (Avihingsanon et al. 2002), acute renal injury (Schaaf et al. 2002; Yoneya et al. 2002), as well as others.

The hemodialysed uremic patients suffer from a periodic oxidative stress (three times a week), which occurs due to the cell shearing and hemolysis during HD. The considerable amount of hem damages the vascular endothelium and contributes to atherosclerosis of these patients. This raises the question how the heme oxygenase reacts to this elevated stress condition. The HO-1 gene expression had not been

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*In memory of Professor Béla Matkovics

studied yet in uremic patients; therefore we wanted to investigate the changes in HO-1 activity simultaneously with the usual parameters of oxidative stress, and the correlation of the two systems during HD. It is known that HO-1 is expressed in monocytes, leucocytes and macrophages (Shibahara et al. 1978) therefore we used blood samples for the non-invasive monitoring of metabolites and HO-1 levels. Since HO-1 induction is primarily regulated at the mRNA level of HMOX1 gene transcription it was feasible to develop a method for measuring the mRNA levels.

Materials and Methods

Seventeen patients with end stage renal disease (ESRD) on regular hemodialysis (HD; 9 male and 8 female aged 14,9±3,1) were studied. The distribution of the original nephrological diagnosis in the ESRD group were as follows chronic pyelonephritis with reflux nephropathy 9, interstial nephritis 2, membranoproliferate glomerulonephritis 3, focal segmental glomerulosclerosis 2, rapidly progressive glomerulonephritis 1. All patients had been on antihypertensive therapy with a combination of angiotensin converting enzyme inhibitor and calcium channel blocker because of hypertension. The patients had been on 4-h bicarbonate HD (3 times a week) for 1-5 years. HD was performed with hemophan single-use dialyser. The blood flow rate was 200 ml/min and the dialysate flow rate was 500 ml/min. During HD heparin was used as an anticoagulant, with initial dose of 500 IU, followed by continuous infusion rate of 1000 IU/h. The venous blood samples were collected before, immediately after HD and 48 hours following HD. For mRNA separation 1ml native blood were collected and stabilized in RNA/DNA stabilization Reagent for Blood/Bone Marrow (Roche Diagnostics GmbH, Germany) for biochemical assays 2-2 ml of EDTA and heparinised blood were collected.

Plasma hemoglobin, bilirubin and blood carboxyhemoglobin, methemoglobin levels

For the assay of plasma hemoglobin and bilirubin contents, heparinised plasma samples were diluted 1:40 (v/v) with 5 mmol L^{-1} PBS, pH 7.4, and measured spectrophotometrically according to the method of Winterbourn (1979). Blood carboxyhemoglobin concentration was also measured spectrophotometrically (OSM 3 Hemoximeter, Radiometer, Coppenhagen).

Reduced and oxidized glutathione

Highly sensitive and specific determinations of total and oxidized glutathione concentrations were made by combining previously accepted standard methods (Németh and Boda 1994).

For the assay of total glutathione concentration, 25 μl of whole blood anticoagulated with EDTA was immediately hemolyzed in 2.5 ml cold (4°C) 0.01 M potassium phosphate buffer containing 5 mM EDTA, pH 7.5, and stored at –70°C until further use. For analysis, 25 μl of hemolyzate was added to the standard glutathione assay mixture (final volume 1 ml) in the order indicated: DTNB (0.6 μmol), glutathione reductase (10 μg) and NADPH (0.2 μmol). The combined action of DTNB and NADPH in the presence of glutathione reductase results in a reaction cycle, the rate of which depends on the total concentration of GSH and GSSG recorded spectrophotometrically at 412 nm during the first 6 min. For the determination of GSSG blood was hemolyzed in the phosphate buffer described above previously supplemented with 0.02 mM N-ethylmaleimide (NEM). The reaction of NEM with GSH results in the formation of a stable complex, preventing GSH from participating in the enzymatic assay and from being oxidized to GSSG. As NEM is an inhibitor of glutathione reductase, it was necessary to separate it by gel filtration with Sephadex G-10 before further analysis. The concentrations of the thiols were expressed with reference to hemoglobin (hgb) determined by the cyanmethemoglobin method.

RNA extraction and cRT-PCR experiments

mRNA was extracted from 1ml venous blood using mRNA Isolation Kit for Blood/Bone Marrow (Roche Diagnostics GmbH, Mannheim, Germany). To avoid loss of mRNA due

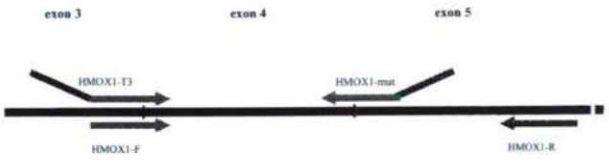

Figure 1. Part of HMOX1 CDS from exon 3 to 5, HMOX1-T3 and HMOX1-mut were used to create a PCR product from which we transcribed our control RNA, which sequence only differs in a 20bp deletion from wild type HMOX1 mRNA. The mRNA and control RNA was reverse transcribed and consecutively amplified by the same primers HMOX1-F and HMOX1-R. Primers were designed to span exon boundaries to avoid binding at genomic DNA level.

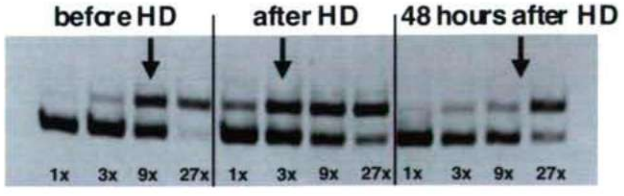

Figure 2. HMOX1 mRNA expressions of a patient with oxidative hemolysis; before, after and 48 hours after HD. The 1, 3, 9, 27x dilutions of control RNA was reverse transcribed with equal amounts of mRNA and amplified by the same PCR primers. The arrows show the concentrations where mRNA concentration is equal to the control RNA concentration.

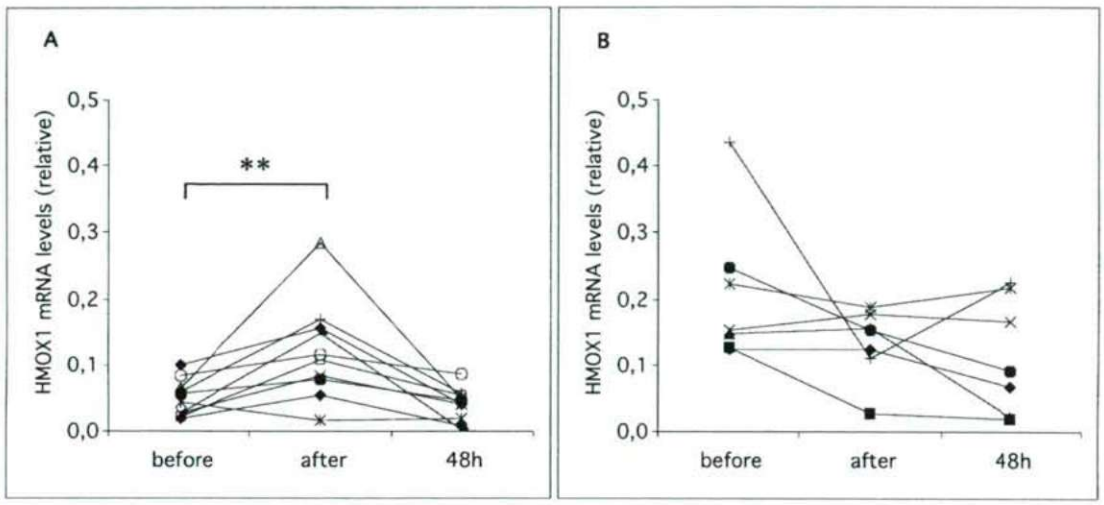

Figure 3. HMOX1 mRNA expression A) in patients with low HMOX1 levels B) in patients with elevated HMOX1 expression.

to limited glass surface or ligands we scaled the separation as we were separating 1.5ml blood samples. Competitive RT-PCR was used to identify the expression of heme oxygenase 1 gene (HMOX1). The competitor RNA was created by in vitro mutagenesis and transcription with T3 RNA polymerase (Fermentas AB, Vilnius, Lithuania) according to Waha et al. (1998). The primers to generate the competitor were as follows: HMOX1-T3: 5' AAT TAA CCC TCA CTA AAG GGA GAC GTT TCT GCT CAA CAT CCA GCT C 3' and HMOX1-mut: 5' CCT GGG AGC GGG TGT TGA GTG GGG GGC AGA ATC TTG CAC TTT G 3'. First strand cDNA was generated using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas AB, Vilnius, Lithuania) using specific primer of HMOX1-R. The specific oligonucleotide primers used for the PCR reaction were designed to span exon boundaries thus binding only at the cDNA level (Fig. 1). The PCR amplification were carried out by the following program: initial denaturation at 94°C for 5 min followed by 25 cycles of denaturation at 94°C for 20 sec, annealing at 61°C for 30 sec and extension at 72°C for 20 sec followed by a final extension at 72°C for 10 min. The primers were as follows: HMOX1-F: 5' CGT TTC TGC TCA ACA TCC AGC TC 3' and HMOX1-R: 5' CCT GGG AGC GGG TGT TGA GTG G 3'. The amplified cDNAs were examined on 6% polyacrylamide gel and stained with ethidium bromide. The target heme oxygenase 1 band was estimated by the ratio to the competitor by densitometry (AlphaImager™, AlphaEase 5.5). The HMOX1 mRNA concentrations were expressed with reference to white blood cell count; since we hadn't calculated the copy number of our control RNA they represent relative values (we used the same dilutions of control RNA). Since control RNA and mRNA was handled in the same tubes together their relative ratio remained the same throughout the whole process.

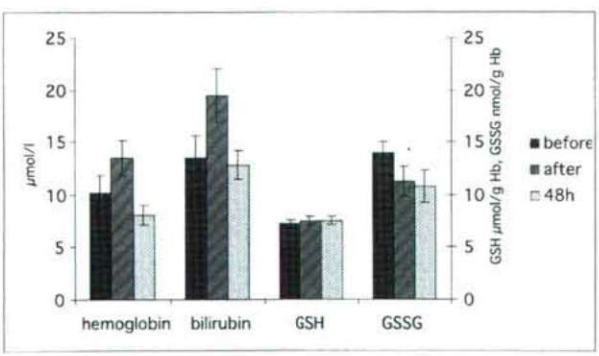

Figure 4. Changes in metabolite levels in the patients (n=10) with low HMOX1 mRNA levels before HD.

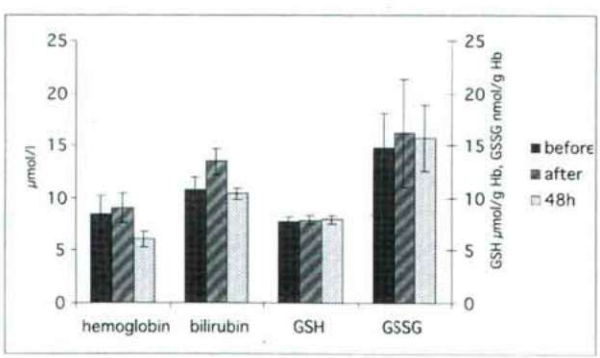

Figure 5. Changes in metabolite levels in the patients (n=7) with elevated HMOX1 mRNA levels before HD.

Statistical analysis

Clinical data on the patients are reported as means ±SD (x ±SD), whereas results of biochemical analyses are shown in figures as means ±standard errors (x ±SEM). Statistical analyses were performed with the Tukey test, Student's *t*-test, and correlation analysis. The significance level for all tests was taken as p=0.05.

Results

We established a cRT-PCR method for the measurement of HMOX1 mRNA levels from blood (Fig. 2). We measured the plasma hemoglobin, plasma bilirubin, carboxyhemoglobin, methemoglobin, GSH and GSSG levels from venous blood samples.

We divided our patients into two groups; in the first group of patients (n=10) the HMOX1 mRNA levels were low before HD. In this group we measured a 1-5-fold increase (p=0.0014) in the HMOX1 gene expression after HD, which dropped back to the original value 48 hours after HD (Fig. 3A). The plasma hemoglobin and bilirubin levels increased after HD and their value dropped back 48 hours after HD (Fig. 4). In the second group there was already a higher level of HMOX1 mRNA expression, in these patients the HMOX1 was not induced further after HD (Fig. 3B). In this group of patients the plasma bilirubin levels increased after HD, and the plasma hemoglobin levels remained the same after HD but both of them decreased 48 hours later (Fig. 5). In the two groups there were no significant changes in the GSH and GSSG levels (Fig. 4,5).

The mRNA levels varied greatly between individuals therefore we calculated the quotient of after HD/before HD of the measured metabolite and HMOX1 mRNA levels, which gives the effect of HD on these parameters.

We found significant correlation between the changes of HMOX1 mRNA levels and plasma hemoglobin (r=0.72, p<0.001) levels, plasma bilirubin (r=0.71, p<0.002) levels compared before and after HD (Table 1, Fig. 6). We found no significant correlation between the changes of HMOX1 mRNA levels and changes in the measured oxidative stress markers (Table 1).

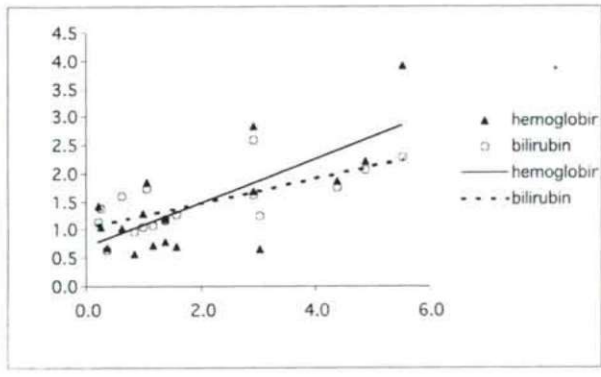

Figure 6. Correlation of the changes in HMOX1 mRNA levels with the changes in hemoglobin, bilirubin levels in all patients (n=17) before and after HD.

Discussion

The average duration of a session for a chronic uremic patient is 4 hours. This time is enough to induce HO-1. Since HO-1 is induced at the mRNA levels the earliest response to the HO-1 induction can be monitored by the measurement of mRNA levels. For the quantitative mRNA analysis we had chosen a cRT-PCR method. This PCR based technique permits the precise analysis from small amount of samples (Kozbor et al. 1993; Waha et al. 1998). The analysis of HMOX1 mRNA from blood samples is noninvasive and easily conducted at the hemodialysed patients. This also gives the advantage that the target tissue (blood) is the same for the measurement of mRNA and other parameters.

In the patients who had already elevated HMOX1 expression before HD the HO-1 catabolyzed the "free" hem and was not further induced. In the patients who had low mRNA levels before HD the HMOX1 was induced by the undergoing hemolysis. The great differences between the base HMOX1 mRNA levels in the patients can be explained by the different redox or inflammatory states (Nath et al. 2001).

Due to the shearing of cells and hemolysis during HD the plasma hemoglobin levels increased, which according to our data and the literature (Nath et al. 2000, 2001; Alam et al. 2002) induced HO-1. In those patients where no marked hemolysis occurred there was no significant HO-1 induction. We did not find any significant changes in the GSH and GSSG levels due to the undergoing HD that confirms our previous data (Túri et al. 1991) and suggest that the glutathione system is not elevated in these conditions.

The effect of HD could be best monitored by the measured parameters after HD and before HD.

The significant correlation between the changes of hemoglobin (inductor) and bilirubin (sequential product) levels with HMOX1 mRNA levels before and after HD shows that hemoglobin induced HO-1, and the induced HO-1 catabolyzed the hem-molecules.

Since we found no correlation between HMOX1 gene

Table 1. Correlation between the changes of biochemical parameters and HMOX1 mRNA levels (r).

biochemical markers	HMOX1 mRNA before HD/after HD
Plasma hemoglobin	0.72; p<0.001
Plasma bilirubin	0.71; p<0.002
GSH	-0.314
GSSG	-0.254
GSH/GSSG	-0.177

expression and the common oxidative markers (GSH, GSSG) our results suggest that the induced HMOX1 expression during hemodialysis is primarily due to the occurring hemolysis.

By our study it is clear that this group of patients has an inducible HMOX1 gene that responds to the stimuli of its substrate and is functionally intact (the induced HMOX1 expression leads to elevated bilirubin level). As we have no hemodialysed control people we don't know whether the induction due to hemolysis in our patients is comparable to normal people. Therefore further studies are needed to investigate a promoter polymorphism – which directly alters the inducibility of HMOX1 gene (Yamada et al. 2001)– on a control population and hemodialysed patients to see whether the HO-1 plays a role in the pathomechanism of vascular damage in uremic patients.

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SYMPOSIUM

Time dependent changes in oxidative metabolism during chronic diabetes in rats+

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ABSTRACT The stability and capacity of antioxidant status during chronic diabetes seriously influence the outcome of the long-term complications caused by oxidative stress. In the present study we investigated the effects of chronic streptozotocin-induced diabetes on the parameters of antioxidant status: activity of scavenging enzymes, gluthation-related and total antioxidant capacity, and degree of lipid peroxidation. Changes in the activities of superoxide-dismutase (SOD), glutathione peroxidase (GSH-Px), gluthatione reductase (GSH-R), and catalase, and in the content of reduced gluthation (GSH), and oxidised gluthatione (GSSG), and in the ratio of GSH/GSSG in blood samples were determined by means of biochemical methods. The degree of lipid peroxidation was measured via thiobarbituric acid assay (TBARS). Hyperglycaemia, ketosis and the accumulation of glycated proteins were estimated by measuring blood glucose, 3-OH-butirate, fructosamine and haemoglobin A_{1c}. In the course of chronic insulin-dependent diabetes, *i.e.* at 2 and 7 days, 10 weeks, and 6 and 8.5 months after streptozotocin injection, hyperglycaemia slightly while ketosis markedly attenuated. Lipid peroxidation was also attenuated. SOD activity decrease in the acute phase only. The activity of GSH-Px increased in the early phase while that of GSH-R mostly decreased in the chronic phase. GSH and GSSG concentrations moved into opposite direction in a time dependent manner. In conclusion, in chronic diabetes an attenuation of severity of diabetes was present throughout the post-injection period, which was well reflected in the improved antioxidant status and capacity.
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KEY WORDS

antioxidant status,
streptozotocin-induced chronic diabetes

Diabetes mellitus is accompanied by hyperglycaemia and a disturbed intracellular carbohydrate metabolism. Hyperglycaemia generates glucose auto-oxidation and auto-oxidative glycosylation of proteins, which may be considered as a combined source of generation of an additional amount of reactive oxygen species (ROS, Baynes 1991; Van Dam et al.1995, 1996). The formation of oxygen free radicals in diabetes results in an extra charge on the antioxidant capacity of the organism and leads to complications in different tissues including vascular bed (Giugliano et al. 1996).

One of the most prominent sources of oxygen free radicals like superoxide and hydroxyl radicals ($^{\bullet}O_2^-$ and $^{\bullet}OH$, respectively) is the process of autooxidation of glucose into enediols in the presence of transition metals (Wolff et al. 1991). The enhanced generation of ROS in diabetes accelerates protein glycation, while glycated proteins become more susceptible to oxidation leading to the accumulation of advanced glycation endproducts (Bonnefont-Rousselot 2002). The ROS-scavenging capacity through the antioxidant systems becomes insufficient in diabetes and a constant oxidative stress develops. Oxidation of lipids, proteins and other macromolecules like DNA become overt with time during the development of diabetes. Lipid peroxides like malonyldialdehyde are the most frequently used compounds for the assessment of the degree of oxidative stress by measuring thiobarbituric acid reactive substances (TBARS, Ohkawa et al. 1979).

In the present study we investigated how the antioxidant capacity in the blood changes during the course of chronic diabetes in rats. Markers of the altered glucose metabolism, *i.e.* blood glucose, 3-OH-butirate, fructosamine and haemoglobin A_{1c} were assayed for estimation of the severity of diabetes. The activities of several scavenging enzymes were also followed. Among them superoxide-dismutase (SOD), glutathione peroxidase (GSH-Px), gluthatione reductase (GSH-R), and catalase were selected. Regarding the endogenous antioxidant defence mechanisms the function of glutathione system was selected and the concentrations of reduced and oxidised glutathione (GSH and GSSG) were measured in the red blood cells (RBC). Changes in concentration of lipid peroxidation products in both RBC and plasma were also estimated as TBARS levels. By this approach a relation between the severity of diabetes and the

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+In memory of Professor Béla Matkovics

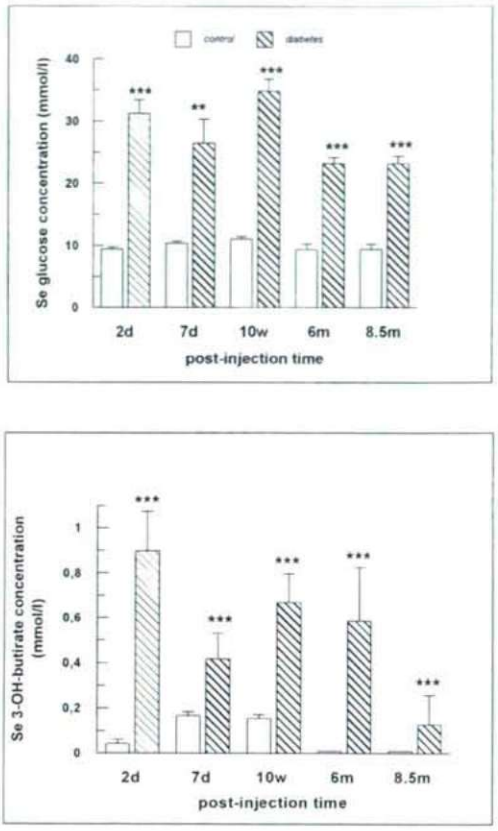


Figure 1. Time course of hyperglycaemia (upper panel) and ketosis (lower panel) in streptozotocin-induced chronic diabetes in rats. ** p<0.01, *** p<0.001 compared to controls (Student's t-test).

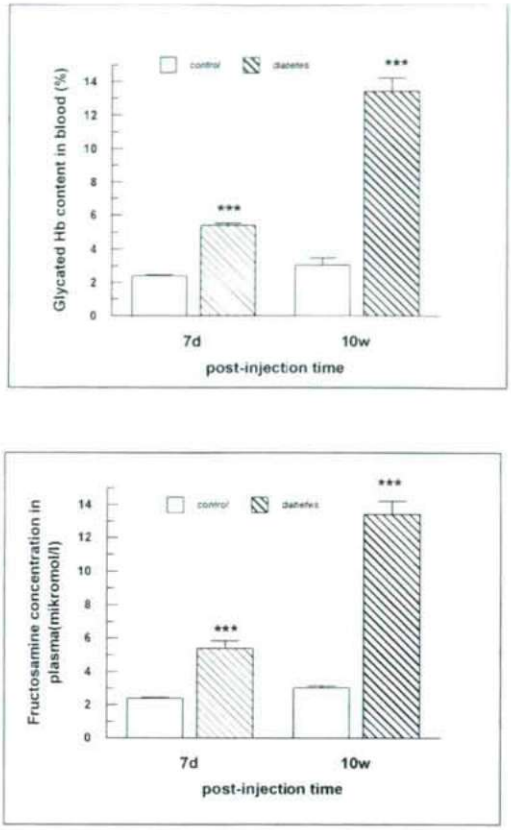


Figure 2. Increment in glycated haemoglobin content (upper panel) and in plasma fructosamine level (lower panel) in rats exposed to streptozotocin injection, during the time of chronic diabetes. *** p<0.001 compared to controls (Student's t-test).

antioxidant capacity of the diabetic organism could be compared.

Materials and Methods

Animals and treatment

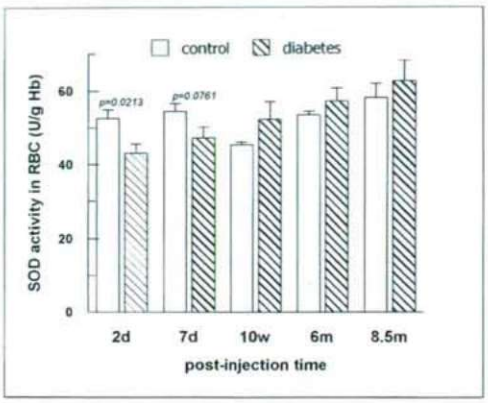
Sixty young adult male Wistar rats (250-300g) were divided into two groups: chronic diabetes and control. The rats were kept under standard laboratory conditions (12/12 h dark/light cycle, lights on at 7.00, room temperature set on 21±1°C, controlled humidity). All experiments on rats were performed in strict compliance with National Institutes of Health Guide for Care and Use of Laboratory Animals (1985).

Diabetes was induced with injection of streptozotocin (Sigma, St. Louis, MO, USA) into the tail vein in a dose of 60 mg/kg body weight. The control rats were injected with saline. Twelve animals were sacrificed after different survival periods at each time, at 2 and 7 days, 10 weeks, 6 and 8.5 months. From the 12 animals 6 were streptozotocin-treated and 6 controls. At the conclusion of the experiment the rats were deeply anaesthetised with chloral hydrate (375 mg/kg body weight; Sigma) and blood was taken from the aorta during exsanguination. Disodium-ethylenediamine-tetraacetate (EDTA, 1 mg/ml, Sigma) was added to blood samples to prevent coagulation. In each case another blood sample was taken without anticoagulation to obtain serum as well. After a short storage at 4°C (maximum 1/2 hour) the anticoagulated blood samples were processed for centrifugation at 3,000 G for 10 min at 4°C. The erythrocytes and plasma fractions were processed separately afterwards. After 3 times washing with ice-cold physiological saline the erythrocyte fraction were hemolysed with distilled water reaching 10X or 2X dilutions and the aliquots were stored at -20°C until biochemical assays.

Biochemical determinations

A) Diabetes markers

To monitor the development of diabetes several variables were measured: serum glucose and fructosamine, plasma 3-hydroxi-butyrate concentration and glycosylated haemoglobin content in the whole blood. Blood glucose concentration from serum was determined spectrophotometrically by a



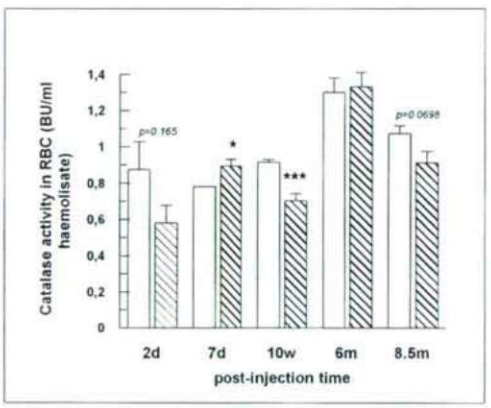
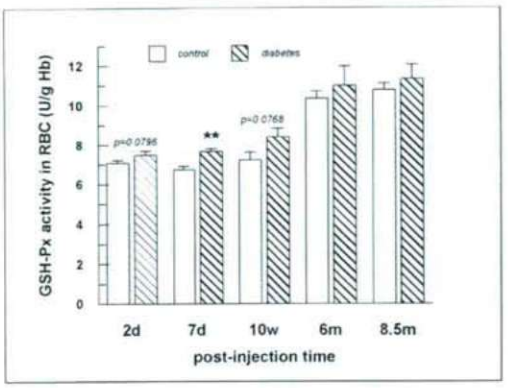


Figure 3. Time dependent changes in red blood cell (RBC) SOD activity (upper panel) and in catalase activity (lower panel) following streptozotocin injection. * p<0.05, *** p <0.001 compared to controls (Student's t-test).



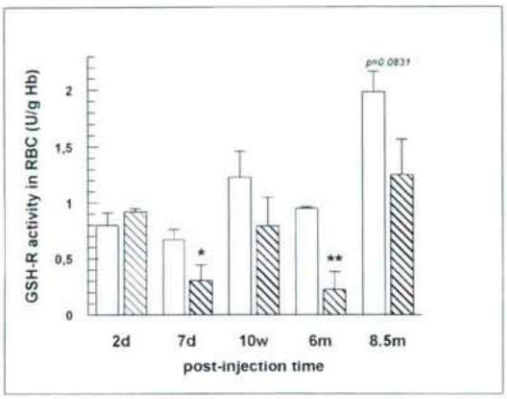


Figure 4. Changes in red blood cell GSH-Px and GSH-R enzyme activities (upper and lower panels, respectively) in the course of chronic diabetes after streptozotocin injection. * p<0.05, ** p<0.01 compared to controls (Student's t-test).

GOD/POD/PAP kit, an enzymatic colorimetric method assaying the reaction product at 505 nm (Trinder et al. 1969). The plasma 3-OH-butyrate concentration was determined by the method of McMurray et al. (1984) by a Randox-kit (United Kingdom). The fuctosamine concentration of the serum was measured by a commercially available kit (Roche). The amount of glycated haemoglobin in whole blood samples was measured after a chromatographic separation on resin with a colorimetric method at 415 nm.

B) Antioxidant parameters

Assaying markers of antioxidant systems the haemoglobin (Hb) content of hemolysates was determined by using MINOS SPE Automate system (Roche, France). Among the antioxidant enzymes the Cu/Zn-SOD activity was determined by the method of Mishra et al. (1972) and Matkovics et al. (1977) based on the inhibition of superoxide ($^{\bullet}O_2^-$)-dependent transformation of epinephrine to adrenochrome. The adrenochrome was measured at 480 nm with Hitachi U-2001 spectrophotometer on standard temperature of 32°C using the 2X-diluted haemolysate. Listing analytical steps briefly, the haemolysate was extracted with chloroform:ethanol = 1:2, the water phase was obtained after centrifugation with 15,000 rpm for 10 min. The activity of SOD was expressed in U/gHb. One unit of SOD activity corresponds to a 50% inhibition of epinephrine-adrenochrome conversion.

Catalase activity of the red blood cells was measured by the method of Beers et al. (1952) by means of monitoring the rate of H_2O_2 consumption at 240 nm on 25°C. The determination was carried out in the 100X-diluted hemolysates. The activity of catalase was expressed in g H_2O_2 hydrolysed/min/ml hemolysate at 25°C.

GSH-Px activity was measured spectrophotometrically by the method of Sedlak et al. (1968; 412 nm; Hitachi U-2001 spectrophotometer) using the 10X-diluted hemolysate by applying 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in the presence of cumene hydroperoxide as a cosubstrate. The activity of GSH-Px was expressed in μmol GSH oxidised/min/gHb at 37°C.

GSH-R activity was determined by the method of Bergmeyer (1963) from the 10X-diluted hemolysate. In this

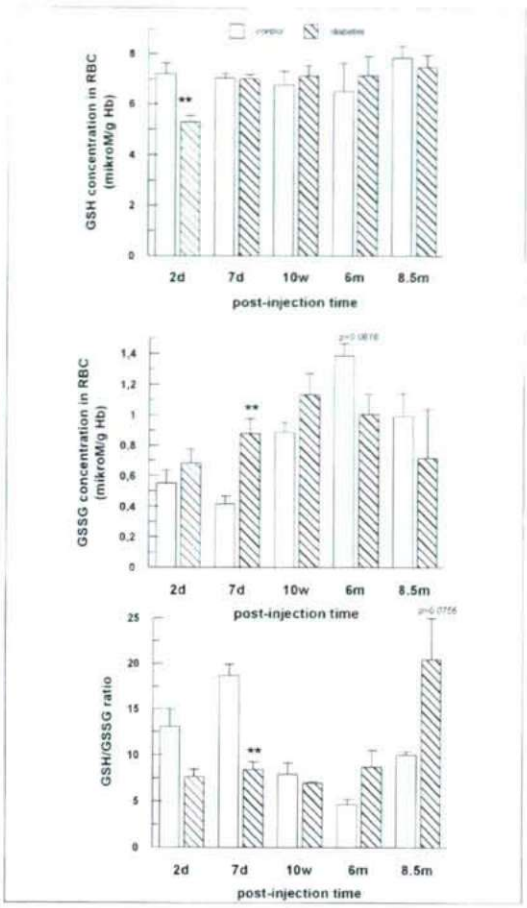


Figure 5. Concentrations and ratio of GSH and GSSG in the red blood cells in the course of chronic diabetes after streptozotocin injection. ** p<0.01 compared to controls (Student's t-test).

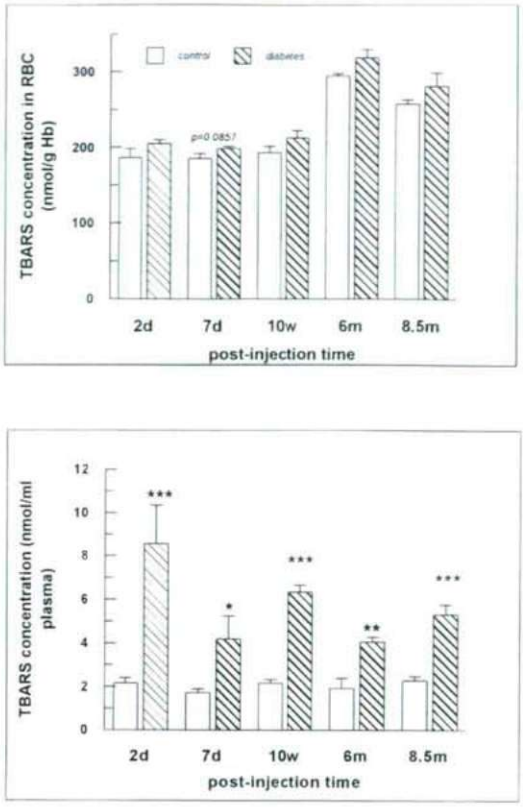


Figure 6. Lipid peroxidation expressed as TBARS concentration in red blood cells (upper panel) and in the plasma (lower panel) in rats exposed to streptozotocin injection. * p<0.05, ** p<0.01, *** p <0.001 compared to controls (Student's t-test).

assay the oxidised form of glutathione is reduced to GSH by GSH-R in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), which serves as a H^+ donor in the chemical reaction. The oxidation of NADPH was followed spectrophotometrically (340 nm, Hitachi U-2001 spectrophotometer) throughout a 3 min period. The activity of GSH-R was expressed in μmol NADPH oxidised/min/gHb at 37°C.

The GSH content of the same hemolysate was measured spectrophotometrically (412 nm, Hitachi U-2001 spectrophotometer), with DTNB, by the method of Beutler et al. (1963) and Tietze (1969). The amount of GSH in the hemolysate was expressed in nmol/gHb.

The determination of oxidised gluthatione in the hemolysate was carried out by the method of Tietze (1969) on 25°C, applying a spectrophotometric measurement with the help of DTNB (412 nm, Hitachi U-2001 spectrophotometer). The amount of GSSG was expressed in nmol/gHb. Finally the ratio of GHS/GSSG was also calculated.

The degree of lipid peroxidation was established by estimation of the thiobarbituric acid reactive substance (TBARS) concentrations in both erythrocytes and plasma. The TBARS content was determined as described by Ohkawa et al. (1979) and Yagi (1976) by using thiobarbituric acid (Merck, Darmstadt, Germany). The TBARS product was assayed by Hitachi U-2001 spectrophotometer at 532 nm. The RBC and plasma TBARS contents were expressed in nmol/gHb and nmol/ml plasma, respectively.

The ferric reducing ability of plasma (FRAP) as a measure of total antioxidant capacity was assayed by the method of Benzie and Strain (1996). The FRAP values was obtained with spectrophotometer measurements at 593 nm.

Results

Characteristics of chronic diabetes

Two days after streptozotocin treatment the blood glucose level increased to the maximum measured throughout this experiment (Fig. 1, upper panel). The serum concentration of 3-OH-butirate was also high on the second day and remained significantly higher during the entire course of the

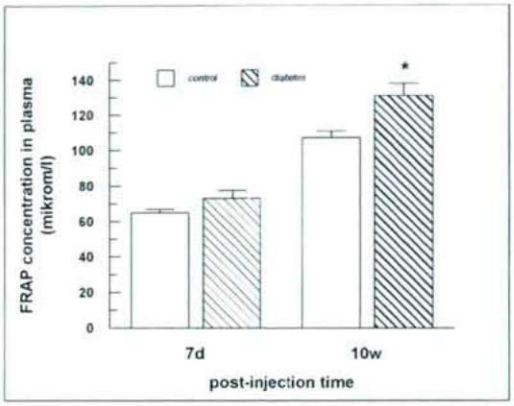


Figure 7. Total antioxidant capacity (FRAP) measured in the plasma of rats exposed to streptozotocin injection. * $p<0.05$ compared to controls (Student's *t*-test).

study (Fig. 1, lower panel). By the end of observation period the glucose level decreased slightly and the 3-OH-butirate concentration showed a more marked diminution, especially at the 8.5 months time point. Those parameters, which reflect accumulation in the molecular effects of chronically high glucose level, *i.e.* glycated haemoglobin and fructosamine, are shown in Figure 2. It is clearly visible that with time the level of both parameters increased significantly.

Parameters of oxidative metabolic state

Activities of oxidative scavenging enzymes are summarised in Figure 3. SOD activity slightly decreased in the acute phase, up to 7 days of the experiment (upper panel). Catalase activity varied throughout the experiment into opposite directions, therefore, no clear change could be realised (lower panel). Enzymes related to the oxidation-reduction circuit of the GSH pathways, GSH-Px and GSH-R, showed also some activity changes (Fig. 4). GSH-Px activity increased significantly at day 7 as well as slightly at 10th week, while other time periods the increment did not reach statistical significance. GSH-R activity, contrary to GSH-Px, performed a decrement from the sub-acute phase (day 7) on.

The concentrations of reduced and oxidised molecular forms of glutathione, *i.e.* GSH and GSSG, as well as their ratio are shown in Figure 5. During the acute phase (day 2) GSH reduced significantly, but the level of this antioxidant marker has been restored from day 7 on (Fig. 5, upper panel). In case of GSSG at the beginning of the observation period the major effect was visible at day 7. This tendency could be seen up to the 10th week, but later the direction of change reversed (Fig. 5, middle panel). The ratio of these two parameters showed a decrease in the acute and sub-acute phases and in the chronic period it tended to be increased (Fig. 5, lower panel).

The degree of lipid peroxidation measured as TBARS level in the RBC and plasma is demonstrated in Figure 6. In the case of RBC a slight tendency of increase was visible throughout of all experimental time-points (upper panel), while in the plasma a marked increment was found in all time periods (lower panel). The highest reaction was visible at day 2 and from day 7 the increment in plasma levels became practically constant.

The parameter of the overall antioxidant capacity reflected in the plasma, *i.e.* the FRAP concentration, slightly increased at day 7 and became significantly more pronounced at the chronic phase.

Discussion

The main findings of this experiment are the followings. (a) After streptozotocin injection in the acute phase of the diabetes (2nd day) blood glucose level showed already a maximum and was accompanied by a maximal production of 3-OH-butirate indicating that ketosis followed the acute diabetes. By the end of the observation period the remarkable decline in 3-OH-butirate indicated that the metabolic severity of diabetes attenuated. (b) FRAP concentration, *i.e.* the total antioxidant capacity showed an increment in the course of diabetes, which is in accordance with the decline in ketosis. (c) The glutathione (GSH) antioxidant system also has been compensated throughout the time-period of chronic diabetes: at the acute and subacute phase the ratio of GSH/GSSG decreased, but in the chronic phase this ratio turned into opposite direction and stayed elevated, indicating that the concentration of the reduced form of glutathione could meet the requirements of the increased oxidative stress. (d) The activities of the scavenging enzymes also changed. SOD declined temporarily but only in the acute phase. GSH-PX activity increased at the acute and subacute phases, while GSH-R activity declined in the advanced period of testing.

To combat ROS the organism has defence mechanisms of enzymatic and non-enzymatic types. The first line of enzymatic defence against the superoxide radical is formed by the superoxide dismutase enzymes (SOD). These enzymes catalyse the diffusion-limited dismutation of superoxide to hydrogen peroxide and oxygen. The cytosolic SOD enzyme contains the metal ions copper and zinc (Cu/Zn-SOD). Hydrogen peroxide in peroxisomes is metabolised by catalase (Oshino and Chance 1977). For further metabolism of cytosolic and mitochondrial hydrogen peroxide a selenium containing scavenger enzyme glutathione peroxidase (GSH-Px) plays an important function. GSH-Px catalyses a reaction in which hydrogen peroxide is reduced to water at the expense of reduced glutathione. The resulting oxidised glutathione molecule is a dimer (GSSG) and is recycled and reduced to GSH by the glutathione reductase enzyme (GSH-R).

In the present study an initial decrement in Cu/Zn-SOD activity was observed in the erythrocytes of diabetic rats (days 2 and 7). By the 10th week and longer periods, however, the activity tended to be higher. It is recognised that oxidative stress inactivates SOD by its enzymatic product H_2O_2 (Salo et al. 1990). At the same time superoxide radical is a signal for increased genetic expression of SOD (Matsuyama et al. 1993). The available amount and activity of SOD, therefore, is the subject of a balance between the production of enzyme molecule and its degradation. As it was shown here this balance shows a time dependent modulation in the course of diabetes.

Two enzymes, GSH-Px and catalase, cooperate in the degradation of H_2O_2. While catalase activity did not show consistent changes, GSH-Px displayed an increment, especially at the acute and subacute phases. It is probable that the increased activity of GSH-Px participated in the gradual but moderate recovery from the acute phase of oxidative stress caused by diabetes. In the very beginning of the diabetic oxidative stress the concentration of GSH decreased, while the oxidised form of glutathione (GSSG) increased. The ration of GSH/GSSG, therefore, decreased at days 2 and 7. In the more advanced periods of the present testing GSSG concentration decreased and the ration of GSH/GSSG tended to be increased. In conclusion, in the course of the observation period the glutathione defence system showed a gradual compensatory increment in its capacity.

The chronic decrement in the activity of GSH-R during prolonged diabetes found in the present study raises several questions. Like SOD the enzymes included in the glutathione redox cycle show susceptibility to oxidation and inactivation. In patients with insulin-dependent diabetes mellitus the GSH pathway enzymes proved to be susceptible to oxidation and this susceptibility increased in poorly controlled diabetics (Dincer et al. 2002). In our present study the activity of GSH-Px was not decreased but only that of GSH-R. It might mean that the GSH redox enzymes are differently sensitive to the destructing action of oxygen radicals.

Lipid peroxidation was studied by measuring TBARS concentration. In the erythrocytes TBARS level increased only moderately but in the plasma a marked elevation could be observed. TBARS concentration in the plasma decreased in the course of observation period with some fluctuation. This finding is in accordance with the idea that there is a functional recovery, at least partial, in the antioxidant defence systems in rats during long-term diabetes.

Acknowledgments

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References

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Schwarz-Sommer Z, Huijser P, Nacken W, Saedler H, Sommer H (1990) Genetic control of flower development by homeotic genes in Antirrhinum majus. Science 250:931-936.

Illustrations

Three complete sets, including a high-quality "original" for publication, must be submitted with the manuscript. The back of each figure or composite plate should be labeled in soft lead pencil, indicating the orientation, the figure number, and the first author's name. The back of the best set should be marked "use for reproduction" or "original". Authors are encouraged to submit digital images of photographs, line drawings or graphs for printing. Most major image editing and drawing/illustrator computer software files (both Macintosh and PC) in TIFF or EPS formats are acceptable. It is particularly important that adequate resolution (at least 300 dpi, preferably 600 dpi) is used in making the original image.

Figure legends

Figures should be numbered consecutively with Arabic numerals. Material in the text should not be duplicated and methods should not be described. The size of scale bars should be indicated when appropriate. The first figure in the text should be referred to as Fig. 1, and so on.

Tables

Tables should be numbered consecutively with Arabic numerals. A brief title should be included above the table. Each table should be printed double spaced, without vertical or horizontal lines, and on a separate sheet. Material in text should not be duplicated and methods should not be described. The first table in the text should be referred to as Table 1, and so on.